Regulation of expression of ULBP1, a ligand for the activating immunoreceptor NKG2D, and its influence on the cytotoxic function of natural killer cells against human leukemia

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Heike Himmelreich
aus Heidenheim a.d. Brenz, Deutschland

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auf Antrag von

Prof. Dr. Ed Palmer
Prof. Dr. Aleksandra Wodnar-Filipowicz
Dr. Helge Grosshans

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Prof. Dr. Eberhard Parlow
Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

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Table of Contents

Abbreviations……..…………………………………………………………………………..8

I. SUMMARY 11

II. INTRODUCTION 13

1. Natural Killer Cells (NK) 13

1.1 NK cell subsets 13

1.2 NK cell receptors 16

1.2.1 Killer immunoglobulin like receptors (KIR) 16

1.2.2 CD94:NKG2 receptors 18

1.2.3 Natural cytotoxicity receptors 18

1.2.4 NKG2D and NKG2D ligands (NKG2D-L) 19

1.3 Effector functions of NK cells 25

2. Acute Myeloid Leukemia (AML) 27

2.1 Risk factors 27

2.2 Diagnosis and classification 28

2.3 Pathogenesis and prognosis 30

2.4 Therapy 33

2.5 NK cells and AML 35

3. Post-transcriptional Regulation of Gene Expression 37

3.1 MicroRNAs 38

3.1.1 MicroRNA biogenesis and mode of action 38

3.1.2 MicroRNAs and cancer 40

3.2.2 MicroRNAs in normal hematopoiesis and acute leukemia 45

3.2 AU-rich elements (ARE) 47

III. AIM OF THE THESIS 50

IV. **RESULTS (PART 1)** **53**

1. **Post-transcriptional regulation of ULBP1, a ligand for the activating immunoreceptor NKG2D** **53**

1.1 Introduction 53

1.2 Material and Methods 56

1.2.1 Cell culture 56

1.2.2 Cloning of luciferase expression vectors 56

1.2.3 Luciferase assay 60

1.2.4 Flow cytometry 60

1.2.5 Quantitative RT-PCR 61

1.2.6 MicroRNA overexpression 62

1.2.7 ULBP1 overexpression 63

1.2.8 Knockdown of Drosha with short hairpin RNA (shRNA) 63

1.3 Results 64

1.3.1 Role of the 3’UTR in regulation of ULBP1 expression 64

1.3.2 Role of ARE in regulation of ULBP1 expression 65

1.3.3 Role of ULBP1-3’UTR fragments in regulation of ULBP1 expression 67

1.3.4 Role of region U1-6 in regulation of ULBP1 70

1.3.5 Role of specific microRNAs in regulation of region U1-6 and U1-9 70

1.4 Discussion 82

1.4.1 Involvement of 3’UTR in regulation of ULBP1 82

1.4.2 Role of ARE in regulation of ULBP1 gene expression 84

1.4.3 Role of microRNAs in regulation of ULBP1 expression 85

1.4.4 Role of the microRNA biogenesis pathway involving Drosha 88

1.4.5 Computational prediction and selection of candidate microRNAs 89

1.4.6 Conclusions 93

V. RESULTS (PART 2) 94

1. Upregulation of NKG2D-L on human fibroblasts upon treatment with stress inducing reagents and histone deacetylase (HDAC) inhibitors 94

1.1 Results 94

1.2 Discussion 97

2. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities 100

2.1 Publication 100

2.2 Supplementary Data 110

VI. APPENDIX 112

1. Manuscript in preparation: Post-transcriptional regulation of ULBP1 ligand for the activating immunoreceptor NKG2D involves 3’ untranslated region 112

2. Figures of the manuscript 132

VII. REFERENCES 138

Curriculum Vitae 151

Abbreviations

3’UTR	3’ Untranslated region
3'RACE-PCR	Rapid amplification of cDNA-ends with polymerase chain reaction
5-FU	5-Fluorouracil
ActD	Actinomycin D
ADCC	Antibody-dependent cell cytotoxicity
Ago	Argonaute
AML	Acute myeloid leukemia
AMO	Anti-microRNA oligonucleotides
AP1	Activator protein 1
APC	Allophycocyanin
ARE	Adenosine/uridine-rich element
ARE-BP	ARE-binding protein
ATM	Ataxia-telangiectasia mutated
ATR	ATM- and Rad3-related
ATRA	All-trans-retinoic acid
B-CLL	B-cell lymphoblastic leukemia
BM	Bone marrow
CBF	Core binding factor
CBP	Creb-binding protein
CD	Cluster of differentiation
Chk2	Checkpoint Kinase 2
CMV	Cytomegalovirus
CPE	Cytoplasmatic polyadenylation elements
CPEB	CPE binding proteins
CPSF	Cleavage and polyadenylation specificity factor
CTL	Cytotoxic T lymphocytes
DAP	DNAX-activating protein
DC	Dendritic cell
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DOX	Doxycycline
DRB	5,6-Dichloro-1-β-D-ribofuranosyl-benzimidazole
eIF4E	Eukaryotic initiation factor 4E

FACS	Fluorescence activated cell sorting
FAB	French-American-British
Fc	Fragment, crystallizable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLT3	FMS-like tyrosine kinase 3
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gemtuzumab ozogamicin
GPI	Glycosylphosphatidyl-inositol
GvHD	Graft versus host disease
GvL	Graft versus leukemia
HA	Haemagglutinins
HCMV	Human cytomegalovirus
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HFF	Human foreskin fibroblasts
HLA	Human Leukocyte Antigene
HSCT	Hematopoietic stem cell transplantation
HSF	Heat shock factor
IFN	Interferon
Ig	Immunoglobuline
IKDC	Interferon-producing killer dendritic cells
IL	Interleukine
IRE	Iron-responsible element
IRE-BP	IRE-binding protein
ITAM	Immunoreceptor tyrosine-based activating motifs
ITD	Internal tandem duplications
ITIM	Immunoreceptor tyrosine-based inhibition motifs
JNK	C-jun NH2-terminal kinase
KIR	Killer cell immunoglobuline-like receptors
LNA	Locked nucleic acid
mAb	Monoclonal antibody
mDC	Monocyte-derived dendritic cells
MDS	Myelodysplastic syndroms
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I

MICA/B	MICA and/or MICB
MIP	Macrophage inflammatory protein
miR	MicroRNA
miRISC	MicroRNA-associated RNA-induced silencing complex
MPD	Myeloproliferative diseases
MULT-1	Mouse UL16-binding protein-like transcript 1
NCR	Natural cytotoxicity receptor
NCR-L	Natural cytotoxicity receptor ligand
NK cell	Natural killer cell
NKG2D	Natural killer group 2, member D
NKG2D-L	NKG2D ligand
NKR	NK cell receptor
NKT	Natural killer T cell
PAP	Poly(A) polymerase
PARN	Poly(A) ribonuclease
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative PCR
RAE	Retinoic acid early
RAET	Retinoic acid early transcript
RARa	Retinoic acid receptor alpha
RIC	Reduced-intensity conditioning
RISC	RNA-induced silencing complex
RL	Renilla
RPL19	Ribosomal protein L19
SEM	Standard error of mean
SHP-1	Src homology 2 domain-containing phosphatase 1
shRNA	Short hairpin RNA
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TRAIL receptor
TSA	Trichostatin A
TTP	Tristetraprolin
ULBP	UL-16 binding protein
VA	Valproic acid
WHO	World Health Organization

I. SUMMARY

Human natural killer (NK) cells are innate immunity $CD56^{+}CD3^{-}$ lymphocytes, mediating spontaneous killing of tumor-transformed cells. The effector functions of NK cells are regulated by a balance of signals initiated from a variety of activating and inhibitory receptors. Recognition of HLA class I molecules on the surface of target cells by inhibitory NK cell receptors (e.g. KIR and NKG2A) ensures that healthy ‚self' cells are protected from NK cell lysis. The loss of HLA class I molecules as a consequence of tumor transformation renders cells susceptible to NK-cell mediated lysis. The cytolytic function of NK cells is dependent on activating receptors, which become engaged by specific cell surface molecules expressed on target cells.

The best characterized activating immunoreceptor is NKG2D, which triggers cellular cytotoxicity and cytokine production upon engagement with its ligands, ULBP and MICA/B molecules. NKG2D ligands (NKG2D-L) are rarely expressed on healthy cells, but frequently upregulated in response to cellular changes caused by malignant transformation. In healthy tissue, inappropriate overexpression of NKG2D-L can trigger autoimmunity. This indicates that NKG2D-L expression has to be tightly regulated to avoid destruction of untransformed tissue, but at the same time to allow recognition and elimination of tumor cells. The molecular mechanisms controlling NKG2D-L expression are poorly understood. Numerous studies demonstrated that different cells and tissues express NKG2D-L transcripts but lack any expression of the corresponding proteins on the cell surface. These findings suggest that NKG2D-L are, at least partly, regulated at post-transcriptional level.

In the first part of the thesis, we examined the involvement of post-transcriptional mechanisms in regulation of the NKG2D-L, ULBP1. Analysis of the 2.4 kb-long ULBP1-3'UTR revealed the presence of four ARE motifs and more then 200 putative microRNA binding sites, regulatory elements which mediate RNA degradation and translational repression. Using luciferase reporter assays, we showed that the full-length 3'UTR of ULBP1 is markedly involved in regulation of ULBP1 gene expression in human cancer cell lines and human primary foreskin fibroblasts. The involvement of ARE elements in negative regulation of ULBP1 gene expression was excluded by mutating ARE motifs in two regions of the ULBP1-3'UTR. The role of candidate microRNAs in regulation of ULBP1 was examined by

mutating the putative microRNA binding sites, or by silencing or overexpression of candidate microRNAs. However, we could neither support nor disprove our hypothesis that microRNAs are involved in regulation of ULBP1 expression, and therefore further studies are needed to elucidate the role of microRNAs. Despite the inconclusive outcome on the microRNA studies, our work provided the first evidence that the regulation of ULBP1 expression takes place on a post-transcriptional level and involves the ULBP1-3'UTR as mediator of RNA destabilization and translational repression.

In the second part of the thesis, we investigated the effect of histone deacetylase inhibitors (HDACi) on surface expression of NKG2D-L in primary human fibroblast (HFF) and AML blasts. Treatment of HFF with trichostatin A (TSA) increased surface expression and transcript level of ULBP1. By using luciferase assay we revealed, that post-transcriptional mechanisms might participate in the upregulation of ULBP1 expression. Treatment of AML blasts with valproic acid (VA) also induced surface expression of NKG2D-L resulting in enhanced killing by NK cells. Efficient cytolysis of AML blasts was achieved by generating alloreactive NK cell lines with KIR-HLA class I mismatch, which allowed to circumvent the signaling by inhibitory NK cell receptors.

Taken together, these data demonstrate, that ULBP1 is regulated by post-transcriptional mechanisms and that the activation of NK cell can be augmented by pharmacological induction of NKG2D-L and the use of alloreactive NK cells.

II. INTRODUCTION

1. Natural Killer Cells (NK)

Human NK cells are crucial components of the innate immune system and play an important role in defense against virus-infected and tumor transformed cells.[1] They comprise about 5 - 15 % of all circulating lymphocytes and are phenotypically characterized by expression of CD56 and lack of CD3 cell surface antigens.[2-3] NK cells are present in peripheral blood, lymph nodes, spleen, bone marrow, liver, peritoneal cavity and placenta.[4-5] In contrast to T cells, NK cells are able to kill target cells rapidly without prior stimulation or exposure to a specific antigen and therefore control infections and tumor growth in an early stage.[6]

1.1 NK cell subsets

Two distinct NK cell subsets (Fig. 1 and 2) can be defined, based on the surface expression of CD56, an isoform of the human neuronal-cell adhesion molecule with unknown function, and the Fcγ receptor III (CD16).[3,7] The majority (~90%) of human NK cells expresses low levels of CD56 and high density of CD16 antigen ($CD56^{dim}CD16^{bright}$ NK cells). These cells are potent effectors mediating antibody-dependent and natural cytotoxicity due to their ability to form conjugates with target cells and their high content of cytolytic granules.[4,8] The cytokine production by $CD56^{dim}$ cells is negligible even following specific stimulation.[7] A minor subset of about 10% of NK cells, displaying a $CD56^{bright}CD16^{dim}$ phenotype, is producing abundant immunoregulatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, TNF-β, interleukine (IL)-10, IL-13, macrophage inflammatory protein (MIP)-1α and granulocyte-macrophage colony-stimulating factor (GM-CSF) upon stimulation with monokines, while exhibiting low cytotoxicity.[9-10] Therefore a major function of $CD56^{bright}$ NK cells might be to provide macrophages and other antigen-presenting cells with IFN-γ and other cytokines for early host defense against a variety of viral, bacterial and parasitic pathogens.[3,9]

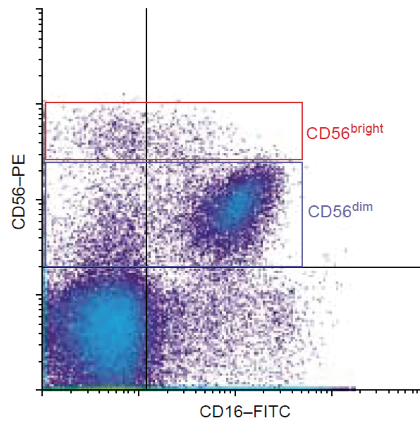

Figure 1: Flow cytometric analysis of CD56^{bright} and CD56^{dim} NK cells.[3] Approximately 90% of all human NK cells are CD56^{dim} NK cell (blue box) and exhibit high-density surface expression of CD16. CD56^{high} NK cells (red box) comprise ~10% of NK cells and express low levels of CD16. Abbreviations: PE, phycoerythrin; FITC, fluorescein isothiocyanate.

In addition, CD56^{dim} cells exhibit high levels of the inhibitory killer cell immunoglobuline-like receptors (KIR) on the cell surface whereas the predominant NK cell receptor (NKR) on CD56^{bright} is CD94/NKG2A.[8-9] Further characteristics of CD56^{dim} cells is the expression of the chemokine receptors CXCR1, CX3CR1 and CXCR4 suggesting that these cells may be attracted to sites of acute inflammation.[11] In contrast, CD56^{bright} cells constitutively express the adhesion molecule L-selectin (CD62L) and the chemokine receptor CCR7 which enables them to migrate through endothelial venules into the lymph node, where they interact with cells of the adaptive immune system.[12] Whereas CD56^{dim} cells express only intermediate affinity IL-2 receptors (IL-2Rβγ) and are therefore weak responders to high doses of IL-2, CD56^{bright} cells additionally express the high affinity IL-2 receptor (IL-2Rαβγ) enabling them to expand in response to low (picomolar) concentrations of IL-2 *in vitro* and *in vivo*.[9,13-14] Triggering of the receptor tyrosine kinase c-kit, exclusively expressed on CD56^{bright} NK cells, augments the IL-2 induced proliferation.[7] Both NK cell subsets constitutively express activating NK cell receptors and receptors for monocyte-derived cytokines including IL-12, IL-15 and IL-18.[3,7,10]

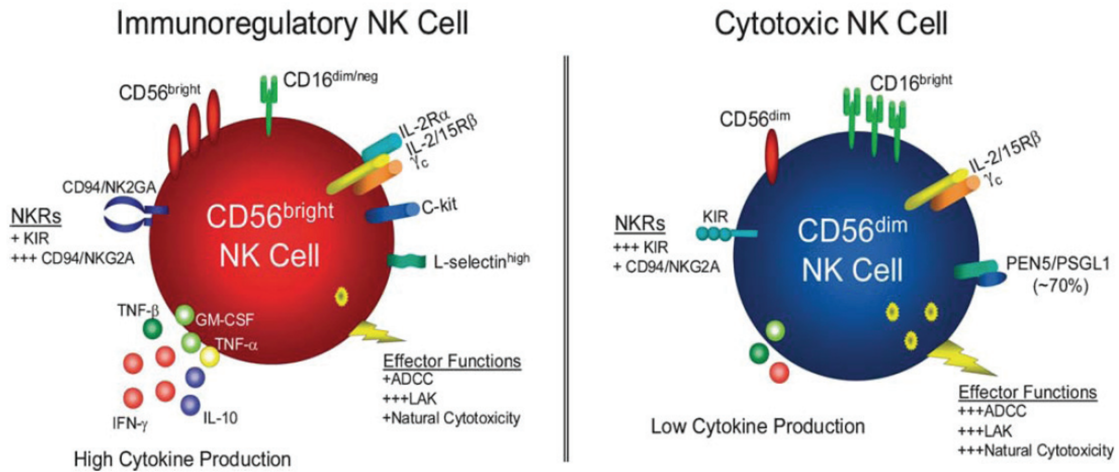

Figure 2: Subsets of human NK cells[7]: The immunoregulatory CD56^{bright}CD16^{dim} NK cells produce high levels of cytokines upon stimulation with monocyte-derived cytokines. Natural cytotoxicity as well as antibody-dependent cellular cytotoxicity is poor. CD56^{dim}CD16^{bright} NK cells are highly cytotoxic and exhibit low production of cytokines.

Table 1: Human activating and inhibitory NK cell receptors and their corresponding ligands

type	activating receptors	ligands	inhibitory receptors	ligands
killer immunoglobulin like receptor	KIR2DS1	HLA-C group 2	KIR2DL1 (CD158a)	HLA-C group 2
	KIR2DS2	HLA-C group 1	KIR2DL2 (CD158b)	HLA-C group 1
	KIR2DL4	HLA-G	KIR2DL3 (CD158b)	HLA-C group 1
	KIR2DS4	unknown	KIR2DL5	unknown
	KIR2DS5	unknown	KIR3DL1 (CD158e)	HLA-Bw4
	KIR3DS1	unknown	KIR3DL2	HLA-A3, -A11
			KIR3DL7	unknown
C-type lectin like	CD94:NKG2C	HLA-E	CD94:NKG2A/B	HLA-E
	CD94:NKG2E/H	HLA-E		
	NKG2D	MICA, MICB, ULBP (RAET)		
Natural Cytotoxicity Receptors	NKp46	viral hemagglutinins, ?		
	NKp44	viral hemagglutinins, ?		
	NKp30	pp65, BAT-3, ?		

1.2 NK cell receptors

NK cell receptors (NKR) are crucial for distinguishing autologous normal cells from transformed or foreign cells. On human NK cells three major subsets of NKRs have been described (Table 1): KIRs and receptors of the C-type lectin superfamily (CD94:NKG2), which both recognize MHC class I and MHC class I-like molecules, as well as natural cytotoxicity receptors (NCRs), whose ligands remain poorly defined.

1.2.1 Killer immunoglobulin like receptors (KIR)

KIRs are a polymorphic set of proteins which are clonally distributed in the NK cell repertoire and recognize HLA-A, -B and -C on target cells.[15] KIRs are structurally characterized by either 2 (KIR2D) or 3 (KIR3D) extracellular immunoglobulin (Ig) like domains. The functional properties of KIRs are determined by the length of their cytoplasmatic tails: KIRs with a long tail (KIR2DL and KIR3DL) mediate an inhibitory signal due to the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM), while the short tail receptors (KIR2DS and KIR3DS) elicit activating signals due to their association with adaptor proteins bearing immunoreceptor tyrosine-based activating motifs (ITAM).[7] All inhibitory KIRs use the same mechanism for signal transduction (Fig. 3), regardless of the diversity of extracellular ligand-binding domains. Binding of MHC class I molecules to KIRs triggers phosphorylation of the ITIM by SRC family kinases and therefore allows binding of the tyrosine phosphatase SRC homology 2 domain-containing phosphatase 1 (SHP-1).[16] In consequence, multiple targets in the ITAM-activating pathway are dephosphorylated by SHP-1 resulting in inhibition of signalling. In contrast, binding of ligands to activating KIRs results in SRC family kinase-mediated phosphorylation of ITAM motifs, located in the associated adapter molecule DAP12 (DNAX-activating protein 12kDa). Subsequent binding of SYK family tyrosine kinases triggers downstream activation cascade.[17]

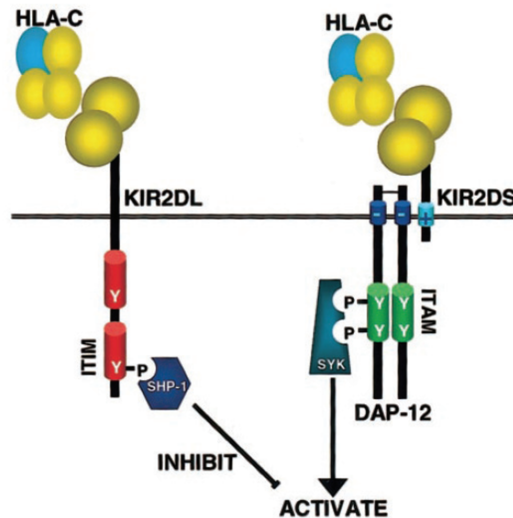

Figure 3: Function of inhibitory and activating KIRs[17]

The highly polymorphic KIR genes are located in the leukocyte receptor cluster on chromosome 19p13.4, one of the most variable regions of the human genome.[18] Based on the content of KIR genes, two KIR hapolotypes, termed A and B, have been defined.[19] Both haplotypes consist of four framework genes: KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1. In A haplotype, four additional genes (KIR2DL1, KIR2DL3, KIR2DS4 and KIR3DL1), which exhibit high allelic variability, are encoded in the genome. Among these, KIR2DS4 is the only activating KIR encoded by the A haplotype. The B haplotype is defined by the presence of the framework genes and one or more genes encoding for the inhibitory KIRs, KIR2DL5A/B and KIR2DL2, and the activating KIRs, KIR2DS1/2/3/5 and KIR3DS1. Thus, the variability in the B haplotype is created by presence or absence of genes, encoding for the inhibitory or activating KIRs, and to less extent by alleles.

The predominant ligands for KIR receptors, involved in the inhibitory regulation of human NK cells, are encoded in the HLA-C locus. Based on the amino acid residues in position 77 and 80 in the $\alpha 1$ helix of the HLA-C molecule, these molecules can be distinguished into two groups, C1 and C2.[17] Group C1 alleles are characterized by Ser77 and Asn80 and are recognized by the inhibitory receptors KIR2DL2 and KIR2DL3, as well as the activating KIR2DS2 and KIR2DS3. Group C2 alleles have Asn77 and Lys80 and bind to inhibitory KIR2DL1 and activating KIR2DS1. Furthermore, the inhibitory receptor KIR3DL1 binds the HLA-Bw4 epitope at amino acid residues 77-83.

1.2.2 CD94:NKG2 receptors

CD94:NKG2 receptors belong to the C-type lectin family and are expressed as heterodimers on the majority of NK cells and a small subset of $CD8^+$ T cells.[20] These receptors are composed of a common subunit CD94 covalently bound to a member of the C-type lectin NKG2 family, and recognize non-classical HLA-E molecules.[21] Since CD94 lacks a cytoplasmic domain for intracellular signal transduction, signalling is mediated through the extracellular and cytoplasmatic domains of the NKG2 molecules.[7] The NKG2 gene family consists of five related transcripts, encoded on chromosome 12, and include NKG2A (and its splice variant NKG2B), NKG2C, NKG2D, NKG2E (and its splice variant NKG2H) and NKG2F.[22] Among these, NKG2D is unique, since it does not associate with CD94, but with DAP10 and DAP12 (see 2.1.4). CD94:NKG2A/B is the only inhibitory receptor of this group and signals through a long intracytoplasmatic tail at the NKG2 subunit, containing ITIM motifs.[23] In contrast, all other NKG2 are composed of short cytoplasmatic tails and mediate activating signals through association with ITAM-containing adapter molecules.[20]

1.2.3 Natural cytotoxicity receptors

The natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 were identified based on their role in natural cytotoxicity towards tumor cells and are exclusively expressed on NK cells.[24-26] Whereas NKp30 and NKp46 are expressed on resting and activated NK cells, NKp44 is induced upon IL-2 stimulation.[25] All NCRs are transmembrane glycoproteins belonging to the Ig superfamily, however share little structural similarity with each other (Fig. 4) and with known human cell surface molecules.[27] Due to lack of signaling motifs in the cytoplamic regions, NCRs associate with the signal transducing adapter proteins CD3ζ, FcεRIγ or DAP12, containing ITAM motifs. This interaction is stabilized through positively charged amino acids in the transmembrane region of the NCRs. Crosslinking of NCRs with monoclonal antibodies induces natural cytotoxicity, cytokine production and Ca^{2+} mobilization. The cellular ligands for NCR are not well defined. NKp44 and NKp46 has been shown to bind viral haemagglutinins (HA) through interaction of NKp44- and NKp46-associated sialic acid residues with sialic acid binding sites of the HA-complex[28-30] Furthermore, heparan sulfate proteoglycans on target cells are recognized by NKp30 and

NKp46 and might be involved in tumor killing.[31] Interestingly, interaction of pp65, a HCMV protein, with NKp30 results in supression of NK cell cytotoxicity.[32] As examined with Fc fusion proteins, ligands of NKp44 and NKp46 (NCR-L) are expressed on malignant melanocytes. [33-34] Furthermore, variable levels of NCR-L are expressed on monocytes and granulocytes of AML patients, whereas malignant blasts are NCR-L low or negative.[35]

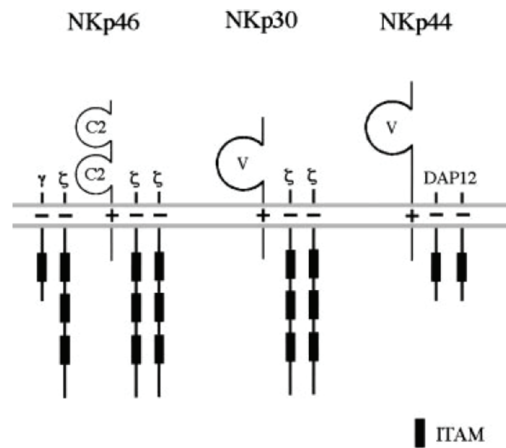

Figure 4: Natural cytotoxicity receptors (modified from[36])

1.2.4 NKG2D and NKG2D ligands (NKG2D-L)

NKG2D receptor

The activating NK cell receptor NKG2D (natural-killer group 2, member D) is a homodimeric lectin like immunoreceptor expressed on NK cells, subsets of T cells (NKT cells, $CD8^+$ T cells, and γδ T cells) and on myeloid-lineage interferon-producing killer dendritic cells (IKDCs).[37-38] Triggering of NKG2D mediates costimulation in cytotoxic T lymphocytes (CTL) and/or activation in NK cells, resulting in proliferation, cytokine production and NK cell cytotoxicity.[39-42]

Signaling of human NKG2D requires noncovalent association with the small adaptor protein DAP10, since NKG2D lacks intracellular signalling domains (Fig.5). Stimulation of NKG2D results in phosphorylation of the YxxM motif in the cytoplasmatic domain of DAP10, and subsequent recruitment and phosphorylation of Phosphoinositide 3-kinase (PI3K) and growth factor receptor-bound protein 2 (Grb2) induces cytotoxicity and IFNγ

production.[37] In mice, two cell type specific splice variants of NKG2D exists: the short form ($NKG2D_{short}$) associates with DAP10, whereas the long form ($NKG2D_{long}$) is capable to associate with DAP10 or the adaptor protein DAP12 (Fig. 5). Signaling via DAP12 involves ITAM and is dependent on the protein tyrosine kinases SYK and/or ZAP-70.[38-39,43]

NKG2D plays an important role in antitumor response. When NKG2D-deficient mice were crossed with transgenic mice spontaneously developing cancer, accelerated tumor growth was observed.[44] Furthermore, enforced expression of NKG2D ligands (NKG2D-L) in tumor cells lead to a potent NKG2D-mediated rejection of tumors *in vitro* and *in vivo*, whereas blocking of NKG2D markely reduced killing of various tumor cell targets. [41,45-49] Neutralizing of NKG2D with an α-NKG2D monoclonal antibody (mAb) enhanced the chemically induced *de novo* formation of fibrosarcoma.[50] However, tumors have developed evasion strategies to circumvent NKG2D dependent immunosurveillance. Tumor cells are able to reduce surface of NKG2D-L by sheding. Subsequent binding of soluble ligands induces endocytosis and degradation of NKG2D in NK cells.[51-52] Chronic exposure of NK cells to ligand expressing tumor cells *in vitro* impaired cytolytic activity against target cells.[53] Furthermore, constitutive local overexpression of the ligands in the epithelial cells or in bone marrow (BM) systemically reduced NKG2D expression *in vivo,* resulting in impaired NK cell function.[54-55]

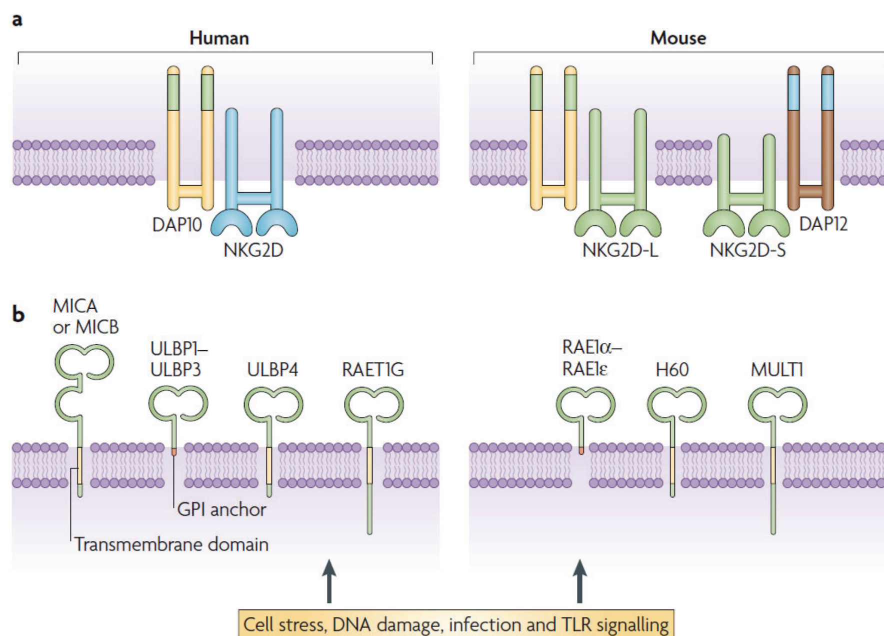

Figure 5: Human and murine NKG2D receptors and their ligands[38]

NKG2D ligands

Human NKG2D ligands (NKG2D-L) are proteins with a structural similarity to MHC class I molecules. In humans, two families of NKG2D-L have been described: major histocompatibility complex class I (MIC)-related molecules and UL-16 binding proteins (ULBP).[38] The MIC family consists of two members, MICA and MICB. MIC genes are highly polymorphic, with over 70 distinct alleles identified, and are encoded in the human MHC locus on chromosome 6q21.[56-57] MIC molecules are expressed as transmembrane glycoproteins and possess $\alpha1$, $\alpha2$, and $\alpha3$ domains, however unlike the classical MHC class I molecules, they do not associate with β_2-microglobulin.[15] The ULBP molecules were identified by their ability to bind to the human cytomegalovirus (HCMV) glycoprotein UL16.[57] To date, six members are identified: ULBP1-4, RAET1G and RAET1L,[56,58-60] which are encoded on chromosome 6q25.[61] ULBPs contain $\alpha1$ and $\alpha2$ domains and, like MIC, do not associate with β_2-microglobulin. ULBP1-3 and RAET1L are glycosylphosphatidyl-inositol (GPI)-linked whereas ULBP4 and RAET1G are transmembrane proteins.[57,61] Murine NKG2D-L consist of at least nine members: five retinoic acid early transcript 1 proteins (RAE-1α-ε), the minor histocompatibility antigen H60, two H60 variants (H60b and H60c) and mouse UL16-binding protein-like transcript 1 (MULT1).[62] All ligands are homologues of ULBP1 and share $\alpha1$ and $\alpha2$ domains. MIC homologues have not been found so far.[63] MULT1 and H60 possess transmembrane domains and cytoplamic tails, whereas RAE-1 molecules are GPI-anchored.[38]

Human NKG2D-L are absent or weakly expressed on normal cells but are upregulated upon viral infection, tumor transformation or cellular stress.[38] Interestingly, transcripts for some ligands are abundantly present in normal tissues, including heart, lung, liver, testis, placenta, kidney, skeletal muscle and tonsils.[60-61,64] MICA and/or MICB (MICA/B) are expressed at low levels on human intestinal epithelial cells, however are upregulated on some epithelial tumors and on HCMV infected endothelial and fibroblast cells.[42,65-67] In healthy individuals, ULBP is absent on T cells, NK cells and erythrocytes, whereas B cells and platelets are ULBP$^+$. On monocytes and granulocytes, expression of ULBP is highly variable among individuals.[68] Bone marrow derived CD34$^+$ progenitor cells are NKG2D-L$^-$ and acquire ligand expression during the course of myeloid differentation.[68] ULBP is expressed on most T cell leukemia cell lines, whereas MIC is absent or expressed at low levels. On cell lines of myeloid and B cell origin, NKG2D-L are not expressed.[69] On primary leukemic cells derived from acute myeloid leukemia (AML) and B-cell chronic lymphoblastic leukemia (B-CLL) patients, low levels of MIC and ULBP molecules were detected.[52,68,70-71] Aberrant

NKG2D-L expression has been also linked with autoimmune diseases, including rheumatoid arthritis, celiac disease and autoimmune diabetes.[72-74] In mice, RaeI and H60 are silent or weakly expressed in adult tissue but frequently induced in tumors and in cells infected with viruses.[47] Abundant levels of MULT-1 transcripts were measured in several tissues, most notably in the thymus and MULT-1 expression occurs frequently on primary lymphomas and adenocarcinomas.[75]

Regulation of NKG2D-L expression

Expression of NKG2D-L must be tightly regulated to ensure elimination of diseased cells while avoiding destruction of healthy cells. Although the molecular mechanisms of NKG2D-L regulation are incompletely understood, recent insights into regulatory mechanisms of NKG2D-L expression have shown, that regulation may take place on transcriptional, post-transcriptional and post-translational levels.

NKG2D-L expression is altered at a transcriptional level in response to several stimuli including heat shock, genotoxic stress, oxidative stress and viral infections (Fig. 6). Retinoic acid (RA) was the first reagent with potential to modulate NKG2D-L expression: transcriptional activity of Rae-1 significantly increased in response to RA in mouse teratocarcinoma cell line and human MIC proteins were upregulated upon treatment with RA in hepatocellular carcinoma cells.[46,76] A RA–inducible element was found in the promoters of Rae-1 family members, suggesting that gene expression is induced at the transcriptional level by RA.[77] Heat shock has been shown to upregulate MICA/B in epithelial cells, due to binding of heat shock factor 1 (HSF1) to the heat shock elements located in the promoter region.[65] In contrast, upregulation of MICA/B in response to CMV infection is not dependent on the promoter region, but requires the expression of the viral intermediate early genes IE1 and IE2.[78] Treatment with agents provoking DNA damage and stalled replication (e.g. 5-FU, aphidicolin, cisplatin and UV-C) activates members of the DNA damage response pathway, ataxia telangiectasia mutated (ATM) or ATM- and Rad3-related (ATR) protein kinases, resulting in increased expression of NKG2D-L in human fibroblasts and mouse epithelial cells.[79] Oxidative stress induced MIC gene expression in colon carcinoma cells.[78,80] Activator protein 1 (AP1), a transcription factor involved in tumorgenesis and cellular stress response was shown to regulate Rae-1ε, since upregulation of Rae-1ε is dependent on low levels of JunB, a member of the AP1 transcription complex, as shown in JunB deficient mouse

embryonic fibroblasts.[81] Finally, NKG2D-L was induced after transfection of the adenovirus serotype 5 (Ad5) E1A oncogene into mouse fibrosarcoma cells resulting in NKG2D-dependent tumor rejection *in vivo*.[82]

Transcriptional regulation of NKG2D-L was also reported for hematopoietic cells. In monocyte-derived dendritic cells (mDC), stimulation of toll-like receptors (TLR) with LPS poly I:C and RNA virus infection increased expression of ULBP1 and ULBP2, resulting in mDC-mediated NK cell activation.[83] Furthermore, *in vitro* maturation of DCs is accompanied by an increase in ULBP1 mRNA and cell surface expression.[84] LPS-dependent upregulation of ULBP1-3 and MICA on the cell surface of human macrophages activated NK cell cytotoxicity resulting in elimination of overstimulated macrophages.[85] Moreover, stimulation of mouse macrophages by TLR ligands induced transcripts of all Rae-1 family members in a MyD88-dependent manner.[86] *In vitro* co-culturing of antigen presenting cells with T cells or treatment of T cells with superantigens increased NKG2D-L expression on T cells and in consequence susceptibility to NK cell lysis.[87-88] Inhibition of ATM and ATR, two members of the DNA damage response pathway, prevented upregulation of MIC in a NFκB-dependent manner.[88] Antitumor reagents have been reported to modulate expression of NKG2D-L. Treatment of primary AML blasts and $CD34^{+}CD38^{-}$ leukemic stem cells with the histone deacetylase inhibitor (HDACi) valproic acid induced surface expression of NKG2D-L resulting in increased susceptibility to NK cell lysis.[70,89] Moreover, low-dose application of the proteasome inhibitor bortezomib enhanced MICA/B expression on hepatocellular carcinoma cells.[90]

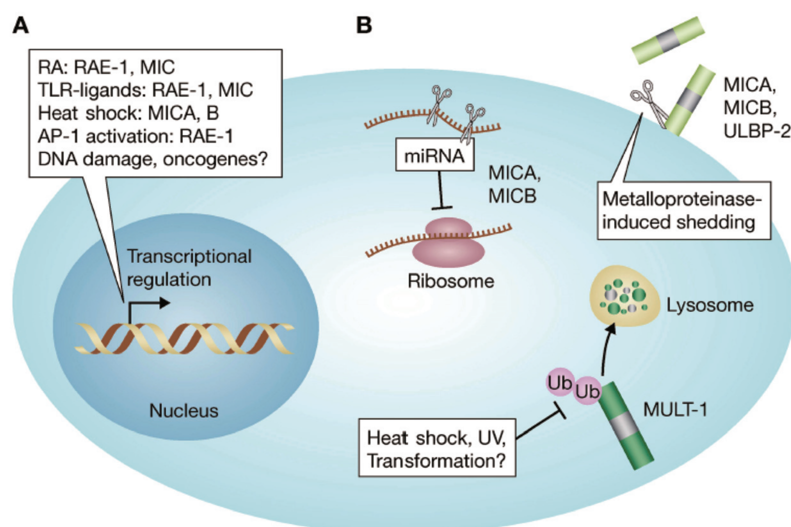

Figure 6: Examples of (A) transcriptional and (B) post-transcriptional regulation of NKG2D ligands[62]

As mentioned above, cell surface receptor expression and mRNA levels of NKG2D-L do not always correlate suggesting that NKG2D-L are at least partly regulated by post-transcriptional mechanisms. Multiple mechanisms of post-transcriptional and post-translational regulation of NKG2D-L such as intracellular retention, microRNA involvement, change of ubiquitination status and proteolytic shedding have been reported (Fig. 6). Infection of cells with HCMV induces transcription of NKG2D-L genes.[42] However, HCMV virus has developed evasion strategies to prevent surface expression of MICB and ULBP1/2 proteins and therefore elimination of infected cells by NK cells: the viral UL16 protein was found to bind these ligands resulting in intracellular sequestration and retention.[91-92] Mandelboim and colleagues revealed another antiviral defense mechanism of HCMV and other herpesviruses. Viral microRNAs influence expression of MICB by targeting the 3'UTR of MICB leading to translational repression and therefore downregulation of this NKG2D-L.[93] Furthermore, also human endogenous microRNAs are able to negatively regulate the translation of MIC ligands.[94-95] IFNγ was reported to decrease the expression of MICA and ULBP2 in melanoma cells and glioma cells and of mouse H60 on sarcoma cells in a STAT1 dependent manner.[96-97] Interestingly, Bui and colleagues showed that the downregulation of MICA on MelJUSo and HeLa cells in response to IFNγ was mediated by a single microRNA, miR-520b, which binds to the MICA promoter region and the 3'UTR.[98] Another mechanism to modulate NKG2D-L post-transcriptionally is proteolytic shedding of MICA, a process that requires metalloproteinases and the cell surface endoplasmatic reticulum 5 protein (ERp5) and might enable the tumor to escape from immunosurveillance.[51,99-100] Finally, the murine NKG2D-L MULT1 was shown to be induced upon heat shock and UV irradiation due to decreased ubiquitination resulting in lysosomal degradation.[75]

This summary shows that numerous groups have investigated the stimuli and the mechanisms, which lead to induction of human and murine NKG2D-L. However one has to emphasize, that many of these studies represent either single reports or results which have not been confirmed by other groups. It appears also that the mechanism of NKG2D-L regulation are most likely dependent on the investigated cell type. Furthermore, most of the results are based on *in vitro* experiments and the relevance of the described mechanisms *in vivo* has still to be elucidated. These facts imply that further studies are needed in order to increase the knowledge about regulation of NKG2D-L expression.

1.3 Effector functions of NK cells

NK cells are potent effectors capable to lyse target cells. The cytotoxicity of NK cells has to be properly controlled to ensure elimination of aberrant cells while sparing healthy cells. The activation of NK cells is tightly regulated by a balance of activating and inhibitory signals mediated by receptor/ligand interactions (Fig. 7).[7] Under normal conditions, elimination of healthy autologous (‚self') cells by NK cells is prevented through recognition of ‚self' MHC class I molecules on target cells by inhibitory NK cell receptors (Fig. 7A).[15] This tolerance to ‚self' requires an education process, before NK cells attain functional competence.[101]Additionally, healthy ‚self' cells mostly lack activating ligands and therefore do not trigger activatory signals. When stimulating signals outweight inhibitory ones and pass a critical threshold, NK cells respond with cytolytic killing and production of cytokines (Fig. 7B).[75] According to the "missing self hypothesis", introduced by Karre and colleagues, ‚self' MHC class I molecules are frequently downregulated upon tumor transformation or viral infection and therefore escape recognition by MHC class I specific NK cell receptors, resulting in elimination of malignant cells by NK cells (Fig 7C).[102] Furthermore, stress and infections can induce cell surface expression of activating ligands. Interaction of these alert molecules with their corresponding receptors results in activation of cell lysis.[7] Watzl and colleagues showed, that inhibitory receptors can abrogate 2B4-mediated activation of NK cells.[103] Antibody-mediated stimulation of the co-stimulatory, activating NK cell receptor NB4 leads to a rapid tyrosine phosphorylation and in consequence to cytotoxicity and IFN-γ release. The NK cell activation was completely blocked due to lack of tyrosine phosphorylation, when KIR2DL1 or CD94:NKG2 were triggered at the same time. Beside receptor/ligand interactions, various other stimuli are able to activate NK cells. Antibody-coated target cells can be recognized by CD16, mainly expressed on $CD56^{dim}$ NK cells, resulting in antibody-dependent cell cytotoxicity (ADCC). Triggering of NK cells by monokines leads to production of IFN-γ and other proinflammatory cytokines/chemokines.[15,104] Furthermore, dendritic cell (DC)-derived cytokines, such as IL-12, IL-18, IL-15 and IFN-α/β, have been shown to promote IFN-γ production, NK cell proliferation and cytotoxicity *in vitro*.[105]

NK cells can eradicate infected or tumor-transformed cells by two major mechanisms, which both require direct contact between NK and target cells[106]. The first process involves exocytosis of perforin- and granzyme-containing granules, which are released into the

intracellular space. Granzyme enters the target cell either through perforin-mediated cytosolic delivery or through perforin-independent uptake via the mannose 6-phosphate receptor, and subsequently induces apoptotic cell death. The second pathway involves engagement of death receptors (e.g. Fas and TRAIL-R) on target cells by their cognate ligands (FAS-L and TRAIL) on NK cells, resulting in caspase-dependent apoptosis.

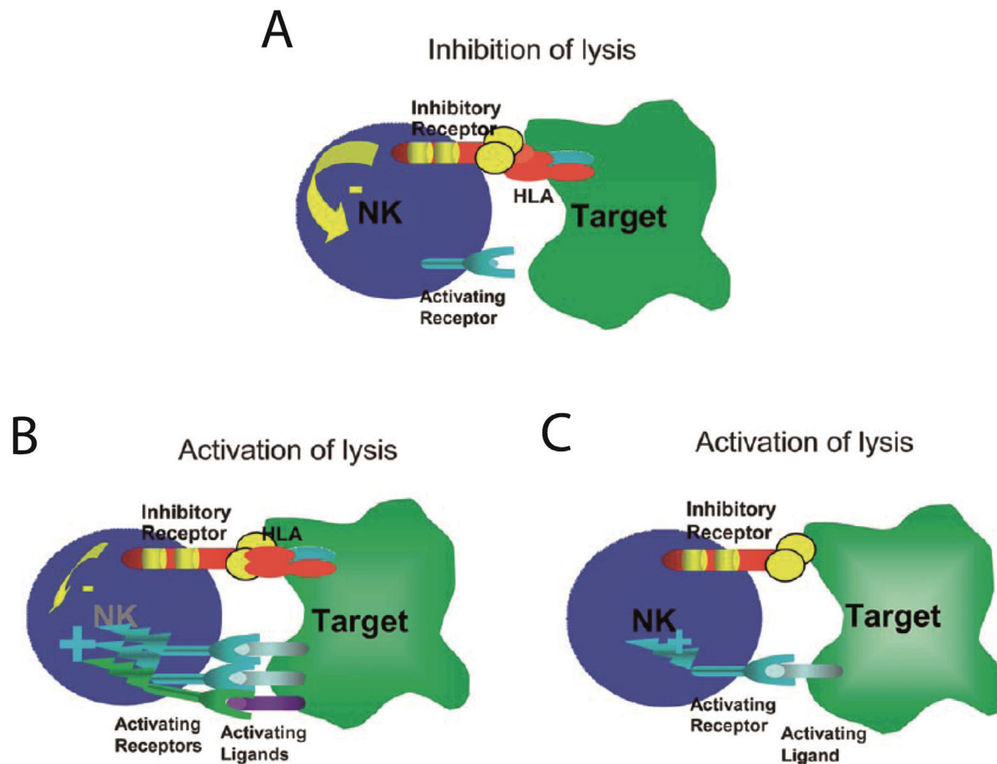

Figure 7: Regulation of NK cell response by activating and inhibitory receptors (modified from[7])

2. Acute Myeloid Leukemia (AML)

Acute myeloid leukemia is characterized by maturation arrest of myeloid progenitor cells in the bone marrow. The deficiency of healthy bone marrow progenitors and their replacement by malignant blasts leads to a reduced number of healthy mature blood cells, causing various clinical symptoms like infections fatigue, paleness, shortness of breath, hemorrhage and fewer. AML is the most common leukemia with an annual incidence of 3.5 per 100000 affecting mainly adults.[107] The annual incidence increases dramatically to 16 per 100000 in persons aged 65 and older. The median age of patients at diagnosis is 67 years. The 5-year survival for all AML patients is 23.4%, and 36.9% for patients younger than 65 years. Although overall survival has increased in the last years through improvement of diagnostic and therapeutic concepts, mortality is still very high and new strategies for treatment of AML are required.

2.1 Risk factors

The majority of AML patients have no known risk factors for developing AML and are therefore considered to have primary AML. However some patients develop secondary AML as a consequence of chemical exposure, ionizing irradiation or prior blood disorders. Known chemicals, which have been associated with development of AML, are chemotherapeutic drugs (e.g. alkylating agents, anthracyclines or topoisomerase inhibitors), benzene and tobacco smoke, which most probably cause various chromosomal aberrations in leukemic cells.[108-111]. Another risk factor for AML is ionizing irradiation. Patients treated with radiotherapy have a risk for developing therapy related AML.[111] Among survivors of atomic bombing in Japan, nuclear tests in the USA as well as radioactive fallout after failures in nuclear power plants, the frequency of developing AML was increased.[112-113] Also chronic exposure to irradiation of jet cockpit crew members and radiologists, employed prior to adoption of modern safety practices, was associated with a significantly increased risk of AML.[114-115] Patients with preleukemic blood disorders like myelodysplastic syndroms (MDS) or myeloproliferative diseases (MPD) have an increased incidence of transforming into AML.[116]

Although AML develops in most cases sporadically due to acquisition of somatic mutations, some cases of familial leukemia have been reported, characterized by autosomal dominant inheritance and a declining age of onset with each generation.[117] A family history of leukemia in a first degree relative increases the risk for leukemia by approximately three- to five-fold and the concordance for leukemia among identical twins is high.[118] Additionally, some congenital diseases exist which have leukemia as a component feature. Individuals with Down syndrome, characterized by trisomy of chromosome 21, have a 10-18-fold increased risk for leukemia and also autosomal recessive syndromes of DNA repair deficiency (e.g. Bloom's syndrome, Ataxia telangiectasia and Fanconi anemia) are predisposed to hematologic neoplasms.[118-119]

2.2 Diagnosis and classification

Modern diagnostic is based on combination of morphologic and cytochemical techniques as well as immunophenotyping by flow cytometry, cytogenetics and molecular diagnostic. The primary diagnostic includes morphologic identification of leukemic blasts (Fig. 8). Since the main characteristic of AML is maturation arrest of immature progenitor cells at different stages of myeloid development, leukemic cells from blood or bone marrow are morphologically characterized according to lineage maturation stage. Based on morphologic appearance of blasts and cytochemistry, the French-American-British (FAB) group has divided AML into nine distinct subtypes M0 – M7 (Table 2).[120] Following the FAB system, a diagnosis is confirmed when the bone marrow contains more then 30% of blasts.

The current schemata for AML classification is the World Health Organization (WHO) system, which divides the disease entities not only morphologically but uses in addition all available information such as cytochemistry, immunophenotype, genetics and clinical features.[121-122] Four major subgroups of AML were defined: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) therapy-related myeloid neoplasms and, (4) AML not otherwise specified. Furthermore, additional minor AML entities exit. The blast threshold for the diagnosis of AML was reduced from 30% to 20% in the blood or marrow, and patients with the particular clonal, recurring cytogenetic abnormalities are considered to have AML regardless of the blast content.

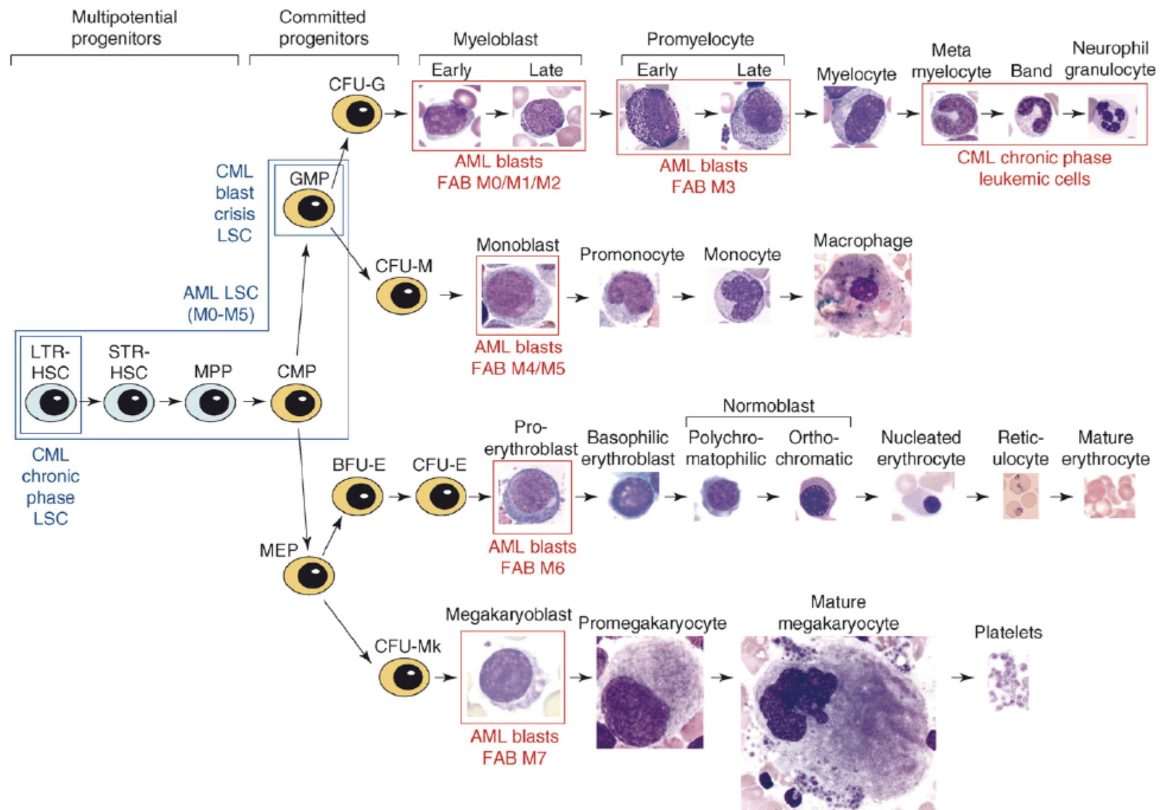

Figure 8: Scheme of normal myeloid development and a relationship to chronic and acute myeloid leukemia[123]

Table 2: French-American-British (FAB) classification of acute myeloid leukemia and associated genetic abnormalities[124]

FAB Subtype	Common Name (% of Cases)	Results of Staining: Myeloperoxidase	Results of Staining: Sudan Black	Results of Staining: Nonspecific Esterase	Associated Translocations and Rearrangements (% of Cases)	Genes Involved
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	−	−	−*	inv(3q26) and t(3;3) (1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15–20%)	+	+	−		
M2	Acute myeloblastic leukemia with maturation (25–30%)	+	+	−	t(8;21) (40%), t(6;9) (1%)	*AML1-ETO, DEK-CAN*
M3	Acute promyelocytic leukemia (5–10%)	+	+	−	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	*PML-RARα, PLZF-RARα, NPM RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23 (20%), inv(3q26) and t(3;3) (3%), t(6;9) (1%)	*MLL, DEK-CAN, EVI1*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)	+	+	+	inv(16), t(16;16) (80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2–9%)	−	−	+	11q23 (20%), t(8;16) (2%)	*MLL, MOZ-CBP*
M6	Erythroleukemia (3–5%)	+	+	−		
M7	Acute megakaryocytic leukemia (3–12%)	−	−	+†	t(1;22) (5%)	Unknown

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).

†Cells are positive for α-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate.

2.3 Pathogenesis and prognosis

AML is characterized by aquisition of somatic mutations in hematopoietic progenitor cells. 55% of adults with AML harbour chromosomal aberrations (e.g. reciprocal translocations, inversions, insertions, deletions, trisomies and monosomies).[125] 45% of patients have a normal karyotype, however carry other genetic lesions like point mutations. Understanding of genetic causes for AML has lead to better prediction of clinical outcome, improvement in classification of AML subgroups, prediction of therapeutic response and to the development of novel therapies that target some of the genetic lesions.

Development of leukemia is thought to happen in a multistep process.[126-127] It has been shown, that expression of a single mutant gene is not sufficient to cause AML and that different mutations cooperate with each other.[128-131] Two classes of mutations can be distinguished (Fig. 9). Class I mutations activate members of signal transduction pathways (e.g. tyrosine kinases FLT3 and c-KIT, N-RAS, K-RAS) resulting in enhanced proliferation and/or survival of hematopoietic progenitors. Class II mutations are often ‚loss of function' mutations affecting transcription factors or components of the transcriptional co-activation complexes. An impaired hematopoietic differentiation and/or aquisition of aberrant self-renewal properties of hematopoietic progenitors is the consequence. Multiple mutations belonging to one of these complementation groups occur rarely in the same patient, whereas mutations between complementation groups often occur together causing an acute leukemia phenotype.

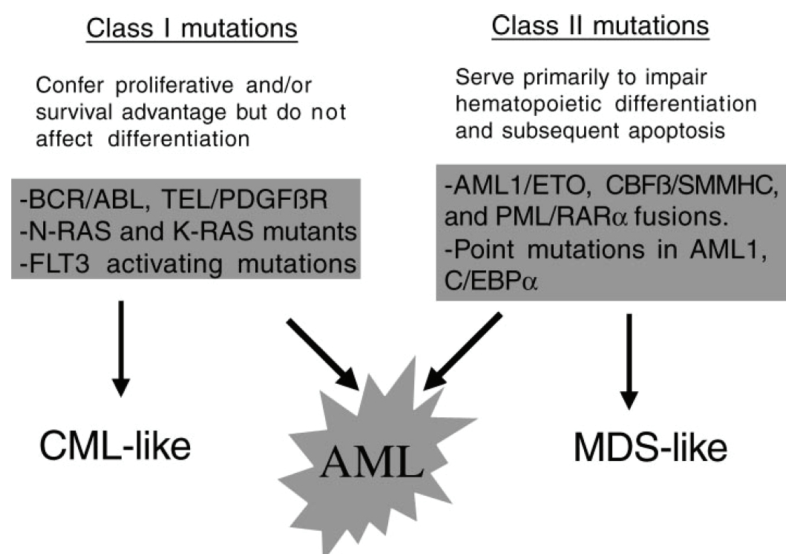

Figure 9: Multistep pathogenesis of AML.[127]

Prevalent types of aberrations are translocations and inversions resulting in generation of fusion proteins. Chromosomal breakpoints are often located in genes encoding for transcription factors (e.g. core binding factor (CBF), retinoic acid receptor alpha (RARα) and members of the HOX family) and for co-activators of transcription such as Creb-binding protein (CBP), p300, MOZ, TIF2 and MLL.[127] Among the most common translocations are t[8;21], inv[16] and t[12;21], resulting in AML1/ETO, CBFβ/SMMHC and TEL/AML1 fusions, respectively.[132-134] AML1/ETO fusion is found in approximately 40% of all AML FAB M2 without beeing restricted to this subtype. AML1, the DNA-binding α-subunit of the transcription factor CBP activates, in combination with its β-subunit CBPβ and other co-activators, the expression of genes essential for hematopoietic differentiation (Fig 10 A). Fusion of AML1 to ETO retains the ability to bind to the target sequence and to interact with CBPβ (Fig 10 B). However, ETO binds to a nuclear co-repressor, resulting in repression of transcription and therefore block of differentiation.[124]

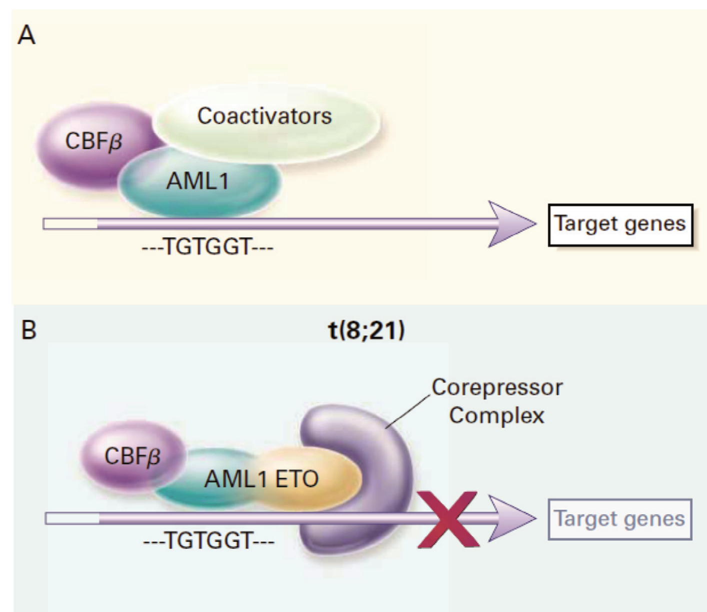

Figure 10: The fusion protein AML1/ETO and its impact on transcription (modified from [124])

Likewise, CBFβ/SMMHC and TEL/AML1 are dominant negative inhibitors of CBF-mediated transcription.[127] Translocation t[15;17] is the cause of the chimeric protein PML/RARα resulting in disruption of development at a promyelocytic stage due to aberrant repression of transcription: While the binding of retinoic acid (RA) to wildtype RARα results in dissociation of the co-repressors SMRT and N-CoR and binding of co-activators, and in

consequence allows the differentiation and granulopoiesis, binding of RA to PML/RARα requires much higher pharmacological doses to dissociate from corepressors.[135] Treatment of leukemic blasts with all-trans-retinoic acid (ATRA) targets PML/RARα and induces differentiation into mature granulocytes.[136] The most frequent genetic aberration on molecular level are mutations in the tyrosine kinase receptor FLT3.[137] In approximately 25% of adult AML patients internal tandem duplications (ITD) are found, with highest incidence in FAB M3 subtype. FLT3 is important for normal hematopoiesis and development of the immune system. Mutations of FLT3 cause autophosphorylation of the receptor, resulting in activation of downstream signalling pathways involved in regulation of transcription, proliferation and apoptosis, an event associated with poor prognosis.[138-139]

The assessment of chromosomal abnormalities in combination with other clinical and laboratory data has allowed to subdivide AML into three prognostic groups: favorable, standard/intermediate, and unfavorable. One important factor to predict the outcome of AML is the cytogenetic status. In addition, other clinical and biological parameters are used to predict the response to treatment and the likelihood to relapse (Table 3).[124,126]

Table 3: Adverse Prognostic Factors in patients with AML (modified from [124])

Factors used to predict response to induction chemotherpy	Factors Used to predict relapse
Unfavorable karyotype	Unfavorable karyotype
Age > 60 yrs	Age > 60 yrs
Secondary AML	Delayed response to induction therapy
Poor performance score	Features of multidrug resistance
Features of multidrug resistance	White-cell count of > 20,000/mm^3
White-cell count of > 20,000/mm^3	Female sex
Unfavorable immunophenotype	Elevated lactate dehydrogenase level
CD34 positivity	Autonomous growth of leukemic cells

2.4 Therapy

The treatment of AML comprises two steps: First, induction therapy is performed, aiming to achieve complete remission, which is defined by reduced amout of leukemic blasts in bone marrow (< 5%) and recovery of blood counts in peripheral blood. In a second step, postremission therapy is administered to sustain remission and prevent relapse.[124]

Induction therapy is administered to all newly diagnosed patients except those with myelodysplasia or secondary AML. Through administration of cytarabine and anthracyclines (e.g. daunorubicin, idarubicin or mitoxantrone), complete remission can be induced in 70-80% of patients younger then 60 years of age, whereas elderly patients > 60 years have a less favorable response rate of 40-50%.[140] To increase the rate of complete remission, several new approaches have been tested, e.g. administration of high-dose cytarabine in combination with daunorubicin and usage of additional cytotoxic reagents or modulators of multidrug resistance.[141-142] However, none of the alternative treatments had any convincing advantages compared to standard therapy. Currently, approaches to sensitize leukemic cells with hematopoietic growth factors to improve their susceptibility to cytotoxic chemotherapy are under investigation.[141-142] However, three studies have showed opposing results concerning complete remission and disease-free survival upon administration of G-CSF and GM-CSF, and therefore clinical investigations are ongoing.

After induction of complete remission, three options for postremission therapy exist, either high-dose chemotherapy, or chemoradiotherapy in combination with autologous or allogeneic hematopoietic stem cell transplantation (HSCT).[142] The choice of therapy is dependent on type of AML, age of the patient, general health, response to induction therapy and, if HSCT is considered, the availability of a stem cell donor. If no postremission therapy is administered, virtually all patients will relapse.[143] Patients with favorable-risk AML are generally treated with 3-4 courses of high-dose cytarabine.[141] In a landmark study of this approach, the overall survival rates after four years was 46 percent.[144] An alternative postremission stategy is the combination of chemoradiotherapy HSCT, whereby the entire hematopoietic system is replaced. Sources of stem cells are, beside the bone marrow, cord blood and “mobilized” blood, where the hematopoietic stem cells migrate out of the bone marrow into the periphery upon treatment with cytokines.[145] For patients with favorable or intermediate risk, ablative cytotoxic treatment followed by autologous HSCT (re-infusion of the patients own stem cells) can be considered as alternative option for postremission therapy.

Promising long-time survival rates of 45-55% have been reported.[124] Disadvantages of an autologous HSCT are possible contamination of the graft with residual leukemic cells that might contribute to disease relapse, and the lack of the graft-versus-leukemia effect.[146] However, benefits of this therapeutic approach may outweight the drawbacks under certain clinical indications. Allogeneic HSCT is considered as the most efficient antileukemic treatment. Transplantation of bone marrow from an HLA-matched sibling can cure 50-60% of recipients. The low relapse rate is the result of a combination of marrow-ablative high dose cytotoxic therapy before bone marrow transplantation and an allogenic graft-against-leukemia effect, mediated through alloreactive T and NK cells. However, these beneficial effects are accompanied not only by high treatment related mortality due to the toxicity of drugs, but also by complications of immunosuppression as well as the risk of graft-versus-host disease (GvHD), restricting allogeneic HSCT mainly to patients younger then 60 years.[124] For patients older then 60 years, reduced-intensity conditioning (RIC) prior allogeneic HSCT has been developed to reduce the treatment related mortality.[147] Results of a retrospective study in AML patients <50 years suggest, that the disease-free survival of patients after RIC regimen HSCT was comparable to those patients receiving standard myeloablative allo-HSCT.[148]

During the last years, the investigation of pathogenic mechanisms on molecular and epigenetic level has revealed strategies to directly target disease associated genetic lesions and molecular defects. For treatment of acute promyelocytic leukemia, which is often associated with the PML/RARA fusion protein, ATRA and arsenic trioxide are efficient drugs resulting in survival rates >70%.[149] Furthermore, three novel agents have reached phase 3 clinical trials and are used in combination with conventional cytotoxic therapy.[141] Gemtuzumab ozogamicin (GO) is a humanized anti-CD33 antibody chemically linked to the cytotoxic agent calicheamicin that inhibits DNA synthesis and induces apoptosis. This drug is approved for relapsed AML in older patients and can produce remissions in 15-35% of these patients.[150] In younger patients addition of GO to standard induction therapy led to a promising 91% complete remission rate.[151] A second group of agents are FLT3 tyrosine kinase inhibitors, which have shown promising response rates in patients with FLT3 mutations.[152-153] The third group of agents are the demethylating agents azacitidine and decitabine. Treatment of AML patients with azacitidine resulted in a 2 year overall survival of 50%, whereas the overall survival in patients treated with conventional therapy was only 16%.[154]

2.5 NK cells and AML

Since NK cells are able to kill myeloid leukemic cells *in vitro* and *in vivo*, these immune effectors might play an important role in immunosurveillance.[89,155-156] The recognition of leukemic cells by NK cells is determined by interactions of KIR and CD94:NKG2A receptors with HLA molecules, resulting in inhibition of NK cell function, and the presence of activating ligands on leukemic cells. Abnormalities in expression of NK cell receptors or ligand in leukemic patients can lead to inadequate NK cell function and in consequence escape of leukemic blasts from NK cell recognition.[157]

Indeed, NK cells of AML patients are altered regarding phenotype and function. Costello and colleagues showed, that NK cells of AML patients are reduced in number and that IL-2 activated NK cells display a low NCR surface density which was accompanied with weak cytotoxicity against autologous leukemic cells.[158]. These results are in contrast to findings of our group who showed, that cytokine-activated NK cell from patients with AML are highly cytotoxic against K562 target cells and are able to produce high amounts of IFN-γ. Expression levels of NKG2D and NKp46 were comparable to activated NK cells from healthy donors.[159] Blasts from most AML patients express low level of ULBPs, MICA/B and NCR-specific ligands and are poorly susceptible to lysis by NK cells.[35,156] The reason for low density of activating ligands might be *in vivo* selection for ligand-low malignant clones, maturation arrest at a ligand low phenotype in hematopoietic differentiation or proteolytic shedding of MIC ligands.[68,99,158] Furthermore, deficient expression of HLA class I molecules on leukemic blasts has been reported.[160-163] Genetic studies revealed, that leukemic patients are associated with a more inhibitory A/B KIR haplotype, compared to controls, and that the frequency of specific inhibitory KIR-HLA class I interactions is increased.[164-165]

Due to their cytotoxic potential against leukemic blasts, NK cells are promising candidates to eradicate malignant cells, which have escaped killing by high-dose chemotherapy. Velardi and colleagues demonstrated that the incidence of leukemic relapse was significantly reduced in AML patients, which have received hematopoietic stem cells from haploidentical donors with KIR ligand incompatibility in graft-versus-host direction. In this situation, alloreactive donor NK cells exhibit cytotoxicity, since inhibitory KIR molecules on the donor NK cell do not become engaged by their corresponding KIR ligand on recipient cells (Fig. 11). In consequence, this alloreactive NK cells are able to mediate graft-versus-leukemia effect against residual leukemic cells.[155]

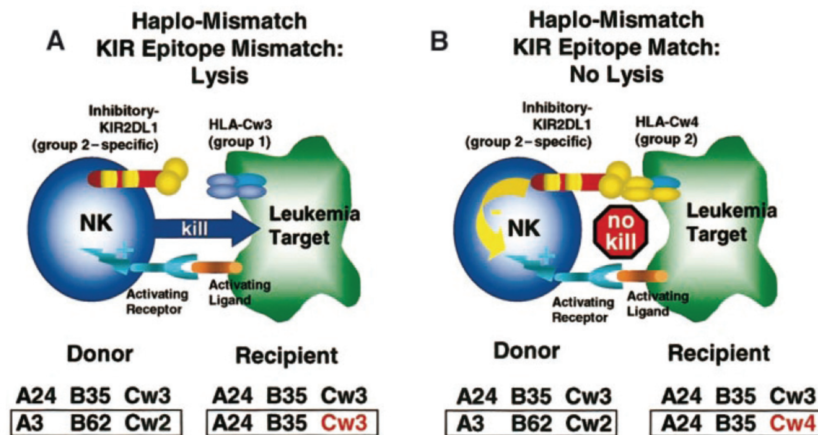

Figure 11: KIR mismatch in haplotype-mismatched stem cell transplantation[166] (A) Donor and recipient are HLA haplotype-mismatched and KIR-mismatched at the HLA-C locus. Donor NK cell clones expressing KIR2DL1 are usually inhibited by ligands of C2 group of HLA-C molecules (HLA-Cw2, 4, 5, 6). However, in this transplantation setting, recipient's leukemic blasts express HLA-Cw3, a member of the C1 group of HLA-C molecules, which is not recognized by the donor's KIR2DL1 receptor, therefore resulting in activation of NK cells and lysis of leukemic blasts. (B) Donor and recipient are HLA haplotype-mismatched but both express HLA-C alleles of the C2 group. Therefore, donor NK cells expressing KIR2DL1 are inhibited by HLA-Cw4 on leukemic cells, resulting in lack of lysis.

Additionally they showed that infusion of alloreactive NK cells into AML-engrafted NOD/SCID mice erradicated human leukemia *in vivo*. Furthermore, infusion of high numbers of allogeneic NK cells into lethally irradiated mice did not cause GvHD and pretransplant infusion of alloreactive NK cells into mice obviated the need for high intensity conditioning to achieve durable full-donor engraftment.[155]

In an autologous or HLA-matched setting, blocking of the interaction between KIR receptors on donor cells and MHC class I molecules on recipient's cells could be a potential therapeutic strategy. In a murine acute leukemia model, blocking of the murine counterpart of inhibitory NK receptors with a monoclonal antibody protected from leukemic death without toxicity. Also adoptive transfer of IL-2 activated NK cells treated *ex vivo* with this antibody significantly increased survival of leukemic mice.[167] Since manipulation of the balance of activating and inhibitory signals in NK cells might open new prospects in immunotherapy, a better understanding of regulation of activating and inhibitory ligands may allow to develop novel therapeutic strategies against cancer.

3. Post-transcriptional Regulation of Gene Expression

The expression of genes is regulated not only on transcriptional level but at multiple steps in the expression pathway including splicing, mRNA transport, polyadenylation, translation, and posttranslational modifications.[168-169] At RNA level, two mechanisms of post-transcriptional regulation are the control of mRNA stability and cytoplasmatic polyadenylation.[170-171] Regulation is accomplished by the interaction of trans-acting RNA binding proteins with cis-acting regulatory elements, which are distributed throughout the transcript. The advantage of regulation at a post-transcriptional level are the possibility to rapidly adjust the protein synthesis to the changes in the cell environment, to fine-tune protein amounts.

Important elements for mRNA stability are the cap structure at the 5'end and the poly(A) tail at the 3'end of the transcript. The poly(A) tail becomes gradually shortened through the deadenylating nuclease. When the poly(A) tail is reduced to a critical length, poly(A) binding proteins cannot bind any longer to the poly(A) tail, which destabilizes the interaction with the 5'cap and translation initiation factors. As a consequence, decapping enzymes remove the exposed cap and the unprotected mRNA is degraded by exonucleases. The rate of poly(A) shortening determines the half-lives of transcripts.[169]

Many regulatory elements are located within the 5'UTR and the 3'UTR of transcripts. A *cis*-element, located in 5'UTR and at the beginning of the coding region of the IL-2 mRNA, has been shown to mediate stabilization of the transcript in response to activation of c-jun NH2-terminal kinase (JNK).[172] The 5'UTR of the ferritin mRNA contains iron-responsible elements (IRE). In response to low iron concentration, IRE-binding proteins (IRE-BP) bind to the IRE and block the ribosome's ability to inititate translation.[173] The coding regions of several messages (e.g. c-FOS and c-MYC) contain specific destabilization elements.[174-175] Poly(A) signals and cytoplasmatic polyadenylation elements (CPE) are specific sequence motifs in the 3'UTR, required for polyadenylation. In oocytes, all CPE-containing mRNAs have short poly(A) tails. Binding of CPE binding proteins (CPEB) to CPEs mediates repression of translation, since the interactions of CPEB with other proteins prevent the assembly of the inititation complex at the 5'end of the mRNA. Upon phosphorylation of CPEB, the cleavage and polyadenylation specificity factor (CPSF) binds to the poly(A) site and interacts with the cytoplasmatic form of the poly(A) polymerase (PAP). After the poly(A) tail is extended, poly(A) binding proteins bind to it and interact with eIF4G, which is together with other initiation factors important for initiation of translation.[171] Two other important

elements, located within the 3'UTR are microRNA binding sites and AU-rich elements, which modulate the mRNA stability positively or negatively. Both elements will be described in more detail in this chapter.

3.1 MicroRNAs

MicroRNAs are short noncoding RNAs ~22 nucleotides in length which play an important role in the regulation of various cellular processes, such as cell cycle, proliferation, apoptosis, differentiation and development. They are expressed in all higher organisms (e.g. humans, animals, flies, worms, plants) and viruses, and function as negative regulators of gene expression through inhibition of translation and/or mRNA degradation.[176-177] MicroRNAs are evolutionary conserved and their genes represent about 1-2% of all human genes. So far, more than 850 human microRNAs are known, which are predicted to regulate 30% of human mRNAs. Single microRNAs are able to target more then 200 mRNAs and, conversely, multiple microRNAs cooperatively control a single mRNA target.[178] MicroRNAs are encoded in the genome as single microRNAs or as multi-cistronic microRNA clusters. The genes are located either in non-coding regions between genes (intergenic), where they are transcribed from their own promoters, or in exons or introns of protein-coding mRNAs and non-coding RNA, where they are usually coordinately expressed with their host gene.[179-181]

3.1.1 MicroRNA biogenesis and mode of action

MicroRNAs are transcribed in the nucleus by RNA polymerase II as large RNA precursors with stem-loop regions, termed pri-microRNAs (Fig. 12).[182] Recognition and cleavage of this molecule by the RNase III enzyme Drosha and its cofactor Pasha/DGCR8 results in generation of ~70 nucleotide-long hairpin intermediates (pre-microRNAs), that are exported to the cytoplasma via the nuclear transport receptor exportin-5 and the cofactor RanGTP. The pre-microRNA is cleaved by the Rnase III enzyme Dicer into double stranded RNA of ~22 nucleotides, and the mature single-stranded microRNA is incorporated into the microRNA-associated RNA-induced silencing complex (miRISC). Recruitment of the microRNAs by the

miRISC complex to microRNA binding sites with fully or partly complementary sequences in the 3'UTR of target genes usually results in gene repression.[181,183] Recent reports suggest that miRNAs also interact with the 5'UTR and open reading frames of genes and mediate not only repression, but also translational activation.[184-186] The precise mechanisms, that lead to silencing of gene expression upon microRNA binding, are so far not well understood. Several published studies suggest, that translational repression might be accomplished though different ways, e.g. inhibition of translational initiation, inhibition of elongation, premature translational termination and cotranslational protein degradation as well as microRNA-mediated decay of target mRNAs.[187]

A crucial factor, determining the mode of action, is the degree of complementarity between the microRNA and the target (Fig. 12).[184] Binding of microRNAs with perfect or nearly perfect complementarity to regulatory elements located in the target transcript induces cleavage by ribonucleases in the miRISC complex, resulting in degradation of the mRNA. This type of mechanism is commonly found in plants, but microRNA-directed cleavage has also been described to occure in mammals. Most animal microRNAs bind with imperfect complementarity to transcripts and repress gene expression post-transcriptionally, resulting in reduction of protein levels without affecting the mRNA levels. However studies have shown, that binding with partial complementarity can also induce mRNA degradation.

The most important nucleotides for microRNA-mRNA interactions are six nucleotides at position 2-7 at the 5'end of the microRNA, called 'seed' sequence.[188] Coherent Watson-Crick base-pairing in the seed sequence is required for binding of microRNAs to their target. Different types of microRNA target sites exist dependent on the number of seed matches (6 or 7 nt), the position of seed matches and the nucleotide composition in the flanking regions. [188] However not only the type of site, but also the number of target sites, the distance between target sites, site position, the local AU content and the 3'pairing of microRNAs influences the efficiency of message destabilization.[188-189]

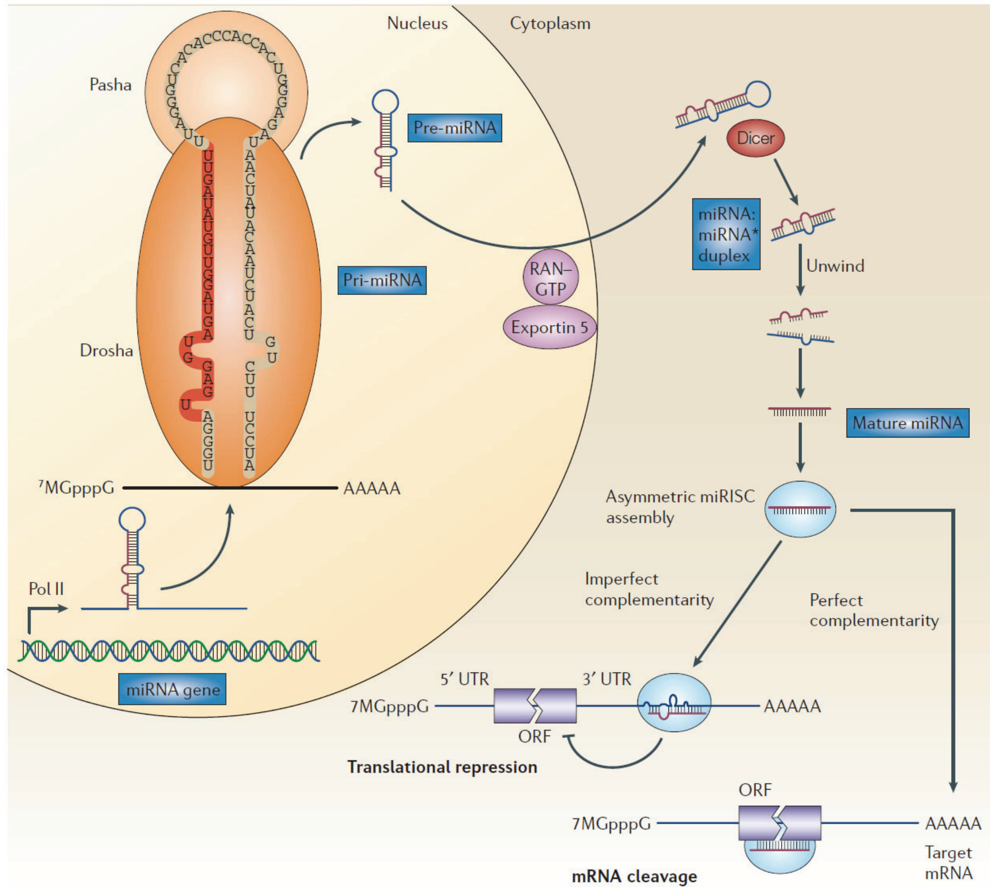

Figure 12: Biogeneis of microRNAs.[190]

3.1.2 MicroRNAs and cancer

Examination of microRNA expression patterns in cancer cells and corresponding healthy tissues revealed that microRNAs are frequently misexpressed in human cancer indicating that microRNAs function as oncogenes or tumor suppressors (Fig. 13).[190] Alterations in microRNA expression may be caused by alterations in the microRNA processing machinery, epigenetic mechanisms or genomic abnormalities (e.g. deletions and mutations, genomic amplifications, chromosomal rearrangements).[191] Many human microRNA genes are located at fragile sites of the chromosome, which are known to be associated with cancer, further supporting the notion, that microRNAs might have important functions in cancer progression.[192]

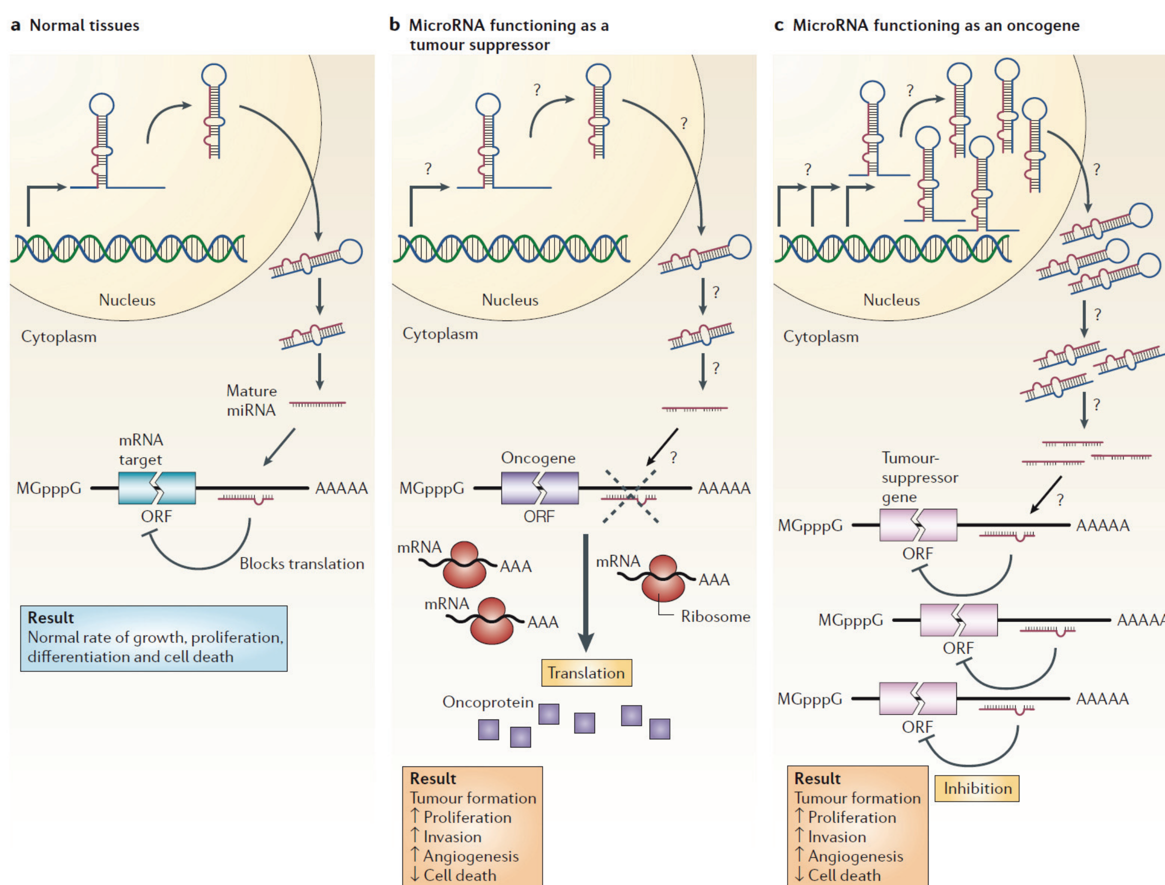

Figure 13: MicroRNAs can function as tumor supressors and oncogenes[190] (a) In normal tissue, correct transcription, processing and binding to target mRNAs lead to translational repression and in consequence to normal rates of growth, proliferation, differentiation and cell death. (b) Defects in any stage of the microRNA biogenesis can lead to elimination or reduction of mature microRNAs that function as tumor suppressors. In consequence miR-target oncogenes are not repressed resulting in tumor formation due to aberrant expression of oncoprotein. (c) Overexpression of microRNAs that function as oncogenes results in tumor formation due to inappropriate negative regulation of miR-target tumor suppressor genes. This misexpression can be caused by amplification of the microRNA gene, a constitutively active promoter of microRNA genes, increased efficiency in microRNA processing or increased stability of the microRNA.

MicroRNAs as tumor suppressors

In human cancer, a global decrease in microRNA level can be observed, indicating that microRNAs act as tumor suppressors.[192-193] First evidence for this model was provided by Calin and colleagues, who described that the miR-15/16-1 cluster, located on chromosome 13q14, is preferentially deleted and downregulated in about 68% of patients with B-cell chronic lymphocytic leukemia (B-CLL).[194] These microRNAs serve as tumor supressors by targeting the antiapoptotic B cell lymphoma 2 (Bcl2) protein.[195] Moreover, the miR-15a/16-1

cluster was shown to regulate survival, proliferation and invasion in a prostate cancer model by targeting CCND1 and WNT3A.[196] Other studies revealed members of the let-7 family as tumor supressors. This microRNA family consists of 12 human homologues, which map to fragile sites associated with lung, breast, urothelial and cervical cancer.[197] The expression of let-7 microRNAs is significantly reduced in lung cancer and correlates with poor prognosis.[198] Let-7 directly regulates important oncogenes, including RAS, MYC and HMGA2, and alters cell cycle, cell division, and cell proliferation genes.[199-202] Furthermore, multiple studies have shown, that microRNAs are important components of the p53 tumor suppressor network. Upon DNA damage or oncogenic stress, p53 transactivates members of the miR-34 family. These microRNAs subsequently regulate cell cycle and DNA damage response genes, resulting in growth arrest and apoptosis.[203-205]

Reduced expression of microRNAs in cancers can be caused not only by mutations in microRNA genes, but also through reduced levels of enzymes crucially required for proper microRNA biogenesis. In ovarian cancer, reduced levels of Dicer and/or Drosha mRNA were observed in ~50% of samples, and low levels of these proteins were associated with poor prognosis.[206] Reduced expression of Dicer was also found in lung cancer cells, and correlated with shorter postoperative survival of patients compared to patients expressing high levels of Dicer.[207] Knockdown of Dicer, Drosha or DGCR8 in murine or human cancer cells resulted in a decreased microRNA levels, and enhanced tumor transformation, accompanied by increased proliferative capacity. Accelerated tumor formation, increased tumor size and invasion into surrounding tissue were observed upon subcutaneous injection of cancer cells, impaired in microRNA-processing, into immunocompromised mice. However, inpairing of the microRNA biogenesis machinery in wild type cells revealed that this event is not sufficient to promote *de novo* transformation.[208]

MicroRNAs as oncogenes

MicroRNAs have also been identified as potential oncogenes, since they can be upregulated in malignant cells. In many lymphomas, the polycistronic miR-17-92 cluster, which includes seven microRNA genes (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR19b-1, miR-20 and miR-92-1), is overexpressed due to amplification. Concurrent overexpression of the miR19-92 cluster and c-MYC accelerated tumor development in a murine B-cell lymphoma model.[209] This microRNA cluster is also markedly overexpressed in lung cancer, resulting in enhanced lung cancer cell growth.[210] Administration of miR-17-5p and miR20a antisense molecules

significantly inhibited cell growth by inducing apoptosis.[211] The oncogenic miR-155 is processed from a precursor form BIC (B-cell integration cluster), a non-coding RNA which accumulates in lymphomas. Expression level of miR-155 is 10 to 30 fold increased in lymphomas compared to normal B cells.[212] B-cell specific overexpression of miR-155 in a transgenic mouse model promoted proliferation of pre-B-cells in spleen and bone marrow, and emergence of high-grade B cell neoplasms.[213] Furthermore, miR-155 is overexpressed in the majority of pancreatic cancers, where it directly regulates the proapoptotic stress-induced p53 target gene TP53INP1.[214] The miR-221/222 cluster is ~11 fold upregulated in thyroid cancer resulting in dramatic loss of KIT transcripts and protein.[215] MiR-211/222 is also overexpressed in glioblastoma and directly targets the tumor suppressor and cyclin-dependent kinase inhibitor CDKNIB/p27^{Kip1}, resulting in continuous proliferation of cancer cells.[216] In hepatocellular carcinoma, high levels of miR-221 negatively regulate both CDKNIB/p27^{Kip1} and CDKNIC/p57.[217] Further, miR-21 is strongly overexpressed in glioblastoma tumor tissue and cell lines, and knockdown of miR-21 in these cells leads to activation of caspases and increased apoptotic cell death.[218]

MicroRNA profiling

Profiling of microRNA expression patterns allows to distinguish cancer tissue from normal tissue through unique microRNA expression signatures. These signatures can be used to classify different tumor types and tumor grades, to cluster sample groups according to their embryonic lineage, to predict prognosis and to determine the specific course of treatment.[193,198,219-221] Expression pattern analysis in six solid cancers (breast, colon, lung, pancreas, prostate and stomach cancer) has revealed that overexpression of miR-21 is a common event in tumorigenesis, suggesting that miR-21 has an important regulatory role in pathways shared by all solid tumors investigated.[222] In colorectal cancer, 28 microRNAs were identified to be differentially expressed compared to normal mucosa, and miR-143 and miR-145 are consistently downregulated in this type of solid cancer.[223] In breast cancer, 29 microRNAs were found to be significantly de-regulated, and among these, a set of 15 microRNAs predicted normal versus cancer tissue with a 100% accurancy.[224] Rosenfeld and colleagues showed, that only 48 microRNA markers are needed to identify the origin of 22 tissues with an accuracy of 90% in patients with metastatic cancer.[225] Therefore, microRNA

signatures can be used to improve diagnosis and prognosis for cancer patients, as summarized in Table 4.

Table 4: microRNA expression profiling in human cancers (adapted from [191])

Cancer type*	MiRNA profiling data	Significance
Chronic lymphocytic leukaemia	A unique signature of 13 genes associated with prognostic factors (ZAP70 and IgVH mutation status) and progression (time from diagnosis to therapy)	MiRNAs as diagnostic markers (the identification of two categories of patients)
Lung adenocarcinoma	Molecular signatures that differ with tumour histology; miRNA profiles correlated with survival (*miR-155* and *let-7*)	MiRNAs as prognostic and diagnostic markers
Breast carcinoma	MiRNA expression correlates with specific pathological features	MiRNAs as prognostic markers
Endocrine pancreatic tumours	A signature that distinguishes endocrine from acinar tumours; the overexpression of *miR-21* is strongly associated with both a high Ki67 proliferation index and the presence of liver metastases	MiRNAs as diagnostic and prognostic markers
Hepatocellular carcinoma	MiRNA expression correlated with differentiation	MiRNAs as prognostic markers
Papillary thyroid carcinoma	MiRNA upregulation (for example, *miR-221* and *miR-222*) in tumoral cells and normal cells adjacent to tumours, but not in normal thyroids without cancers	MiRNAs probably involved in cancer initiation
Glioblastoma	A specific signature compared with normal tissues	MiRNAs as diagnostic markers
Human cancers	MiRNA-expression profiles accurately classify cancers; an miRNA classifier classes poorly differentiated samples better than a messenger RNA classifier	MiRNAs as diagnostic markers
Human solid cancers	Common signature for distinct types of solid carcinomas	Specific miRNAs are involved in common molecular pathways

*Only data from microarray studies reporting results on human primary tumours were included in this table. IgV_H, immunoglobulin heavy-chain variable-region. MiRNA, microRNA. ZAP70, 70 kDa zeta-associated protein.

MicroRNAs as therapeutics

Since microRNAs function as oncogenes and tumor suppressors, they might be targets for cancer therapies. One therapeutic strategy aims to quench oncogenic microRNAs by antisense-mediated inhibition. Knockdown of endogenous onco-miR-21 by synthetic 2'-O-methyl modified anti-microRNA oligonucleotides (AMO) in glioblastoma or breast cancer cells as well as in a xenograft mouse model inhibited cell growth due to increased apoptosis.[218,226] A single intravenous injection of cholesterol-conjugated AMOs to target liver specific miR-122 into mice silenced miR-122 for up to 23 days and decreased the cholesterol serum level by ~40%.[227] Intravenous injection of more stable and less toxic locked nucleic acid (LNA)-based olignonucleotides (LNA-antimiR) into African green monkeys resulted in depletion of mature miR-122 and a dose-dependent and long-lasting lowering of plasma cholesterol.[228] Stable knockdown of microRNAs *in vivo* can be achieved through lentiviral delivery of artificial anti-microRNA decoys, comprised of multiple complementary binding sites for the targeted microRNA. Transplantation of hematopoietic stem cells, containing an

anti-miR-223 decoy, into lethally irradiated mice resulted in functional knockdown of miR-223 that phenocopied the properties of the miR-223 knockout mouse.[229]

Another strategy showing great therapeutic promise is to replace microRNAs by chemically modified double-stranded RNA oligonucleotides, so-called microRNA mimics, or viralvector-encoded microRNAs in tumors with reduced microRNAs expression. Adenovirus-mediated overexpression of let-7 in a murine model of human lung cancer model suppressed lung tumor initiation and resulted in a 66% reduction of tumor burden compared to control mice.[230] Similarly, mice injected with tumor-initiating breast cancer cells, which were transduced with lentiviruses containing let-7 overexpression cassette, developed significantly fewer tumors compared to mice injected with non-modified breast cancer cells.[231] Bonci and colleagues showed that reconstitution of miR-15a and miR-16 expression *in vitro* and *in vivo* induced growth arrest and apoptosis in prostate cancer cells, and lead to considerable volume reduction in a xenograft prostate cancer model.[196] In mice harbouring liver tumors, significant tumor regression was observed after adenoviral delivery of miR-26a.[232]

3.2.2 MicroRNAs in normal hematopoiesis and acute leukemia

MicroRNAs are crucial regulators of many physiological processes including haematopoietic differentiation. As already mentioned in section 2.3.1.2, inappropriate expression of microRNA contributes to leukaemogenesis.

First evidence, that microRNAs play a role during normal hematopoiesis was provided by Chen and colleagues.[233] From ~100 microRNAs, which were cloned from mouse bone marrow, miR-181, miR-223 and miR142s were differentially or preferentially expressed in hematopoietic tissue as well as in individual hematopoietic cell lines suggesting, that these microRNAs influence hematopoietic lineage differentiation. Indeed, ectopic expression of miR-181 in hematopoietic progenitor cells increased the fraction of B cells in *in vitro* differentiation assays and upon transplantation into lethally irradiated mice *in vivo,* indicating that miR-181 is a positive regulator of B-cell differentiation. In mice, miR-150 is expressed in mature lymphocytes, but not in their progenitors.[234] In miR-150 knockout mice, a subclass of B-cells, so-called B1 cells, are expanded in the spleen and in the peritoneal cavity and the amount of antibodies in the blood serum is increased.[235] Ectopic expression of miR-150 in transgenic mice reduced the expression of c-MYB, a transcription factor important for

lymphocyte development, in a dose-dependent manner and dramatically impaired lymphocyte development. The myeloid-specific miR-223 negatively regulates the proliferation of progenitors, as well as granulocyte differentiation and activation.[236] The expression levels of miR-223 are low in hematopoietic stem cells and myeloid progenitors and increase steadily throughout granulocytic differentiation. Mutant mice lacking miR-223 have significantly higher numbers of neutrophils due to increased number of granulocyte progenitors. Further examples of microRNAs involved in hematopoiesis are listed in Table 5.

Table 5: Examples of microRNA function in normal hematopoiesis (adapted from [237])

miRNA	Function in normal hematopoiesis
let-7	represses megakaryocytopoiesis
miR-15 and miR-16	promote erythropoiesis
miR-17-92 cluster	downregulated during monocytopoiesis and megakaryocytopoiesis. Represses monocytopoiesis and megakaryocytopoiesis, and promotes the transition from pro-B to pre-B cell stage
miR-155	Repressed both megakaryopoiesis and erythropoiesis. Important in lymphopoiesis and immune response (for both B and T cells)
miR-196a and miR-196b	In the hematopoietic lineage, reach a peak in short-term repopulating hematopoietic stem cells ans then decrease as cells become more differentiated. Significantly downregulated furing transition from common myeloid progenitors to granulocyte-macrophage progenitors

Examination of microRNA expression pattern in leukemic and healthy hematopoietic cells revealed a number of microRNAs, which are misregulated in leukemia (Fig. 14).[237] Distinct expression patterns were observed for AML subtypes harbouring particular types of cytogenetic and molecular alterations, and selected sets of microRNAs could predict different types of AML with high accuracy.[238-239] In addition, the expression signature of some microRNAs was predictive of outcome and survival of patients with leukemia. The overall and event-free survival was significantly worse in AML patients with high expression of miR-191 and miR-199a than in AML patients with low expression.[240] In AML patients without cytogenetic alterations, the expression of miR-181a and miR-181b was associated with good outcome, whereas the expression of miR-124, miR-128, miR 194, miR-219-5p, miR-220a and miR-320 were associated with poor outcome.[241]

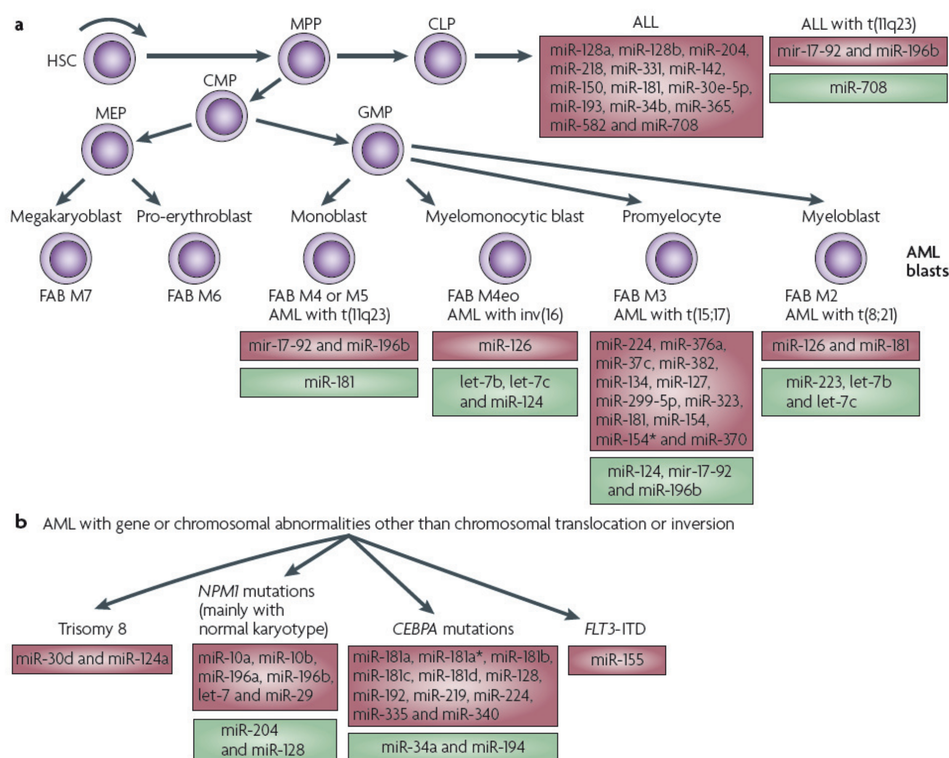

Figure 14: Involvement of microRNAs in acute leukemia[237] Upregulated microRNAs are indicated in red, downregulated microRNAs are indicated in green.

3.2 AU-rich elements (ARE)

AU-rich elements were originally found in the 3'UTR of short-lived mRNAs encoding for proto-oncogenes, growth factors and cytokines. Further studies revealed, that 5-8% of human mRNAs contain ARE. These elements are involved in regulation of mRNA turnover since they promote rapid mRNA decay or stabilization of transcripts. ARE are sequence elements of 50-150 nucleotides that are rich in adenosine and uridine bases.[242] Based on the number and distribution of the pentameric motif AUUUA, ARE can be assigned to three main classes (Table 6).[243] Class I AREs contain one ore more dispersed copies of the AUUUA motif embedded within U-rich regions. Class II AREs consist of at least two overlapping copies of the nonameric sequence UUAUUUA(U/A)(U/A) and can be further subdivided into 5 groups based on the repetition pattern of AUUUA pentamers.[244] Class III AREs lack the typical ARE motifs AUUUA.

Table 6: Classification of ARE sites (modified from [242] and [169])

class	motif	examples
I	1-5 dispersed AUUUA motifs in a U-rich context	c-myc c-fos IFN-γ
IIA	$(AUUU)_5A$	GM-CSF TNF-α
IIB	$(AUUU)_4A$	IFN-α
IIC	$(A/U)(AUUU)_3A(A/U)$	Cox-2 IL-2 IFN-β
IID	$(AUUU)_3A$	FGF2
IIE	AUUUA	u-PA receptor
III	no AUUUA motif, U-rich region	c-jun p53

ARE binding proteins (ARE-BP) are *trans*-acting factors, that bind specificially with high affinity to AUUUA motifs and/or U rich regions in the 3'UTR of transcripts.[243] Interaction of ARE-BPs with ARE can have either negative or positive effect on mRNA stability, translation and subcellular localization.[169] One ARE-BP is able to bind multiple mRNAs and, furthermore, individual mRNAs can bind more than one ARE-BP.[242] Several ARE-BP have been described. HuR belongs to the superfamily of ELAV-related proteins.[242] This ubiquitously expressed protein is predominantly located in the nucleus, however can shuttle between nuclear and cytoplasmatic compartments. HuR is able to bind to all three classes of AREs, resulting in stabilization of transcripts *in vitro* and *in vivo*. Furthermore, HuR serves as an adapter molecule for the nuclear export of c-FOS. AUF1/hnRNPD (heterogeneous nuclear ribonucleoprotein D) is, like HuR, a nuclear protein and can shuttle between the nuclear and cytoplasmatic compartment. AUF1 binds to class I and class II AREs and induced mRNA decay under normal conditions. Upon stress (e.g. heat shock), mRNA stability was shown to be enhanced. Tristetraprolin (TTP) is, in contrast to HuR and AUF1, mainly located in the cytoplasm and targets only class II ARE. TTP was shown to destabilize TNF-α, GM-CSF and IL-2.

The mechanism, how binding of ARE-BP to the transcript alters mRNA decay rates is still unclear. One proposed mechanism is, that ARE-BP influence the interaction of the translation initiation factors elF4E (4E), elF4G and the poly(A)-binding protein (PARB) with the 5'cap structure and the poly(A) tail of the transcripts.[169] During translation these molecules are thought to promote circularization of the mRNA and therefore protect the mRNA from attack of deadenylases and decapping enzymes. Interaction of destabilizing factors, such as AUF1, with ARE elements might alter the interaction of PARB with the poly(A) tail, resulting in accessibility of the poly(A) tail to PARN (poly(A) ribonuclease) and in consequence rapid deadenylation-dependent decay. Conversely, stabilizing ARE-BPs, such as HuR, might enhance the binding affinity of PARB to the poly(A) tail and therefore block deadenylation.[169]

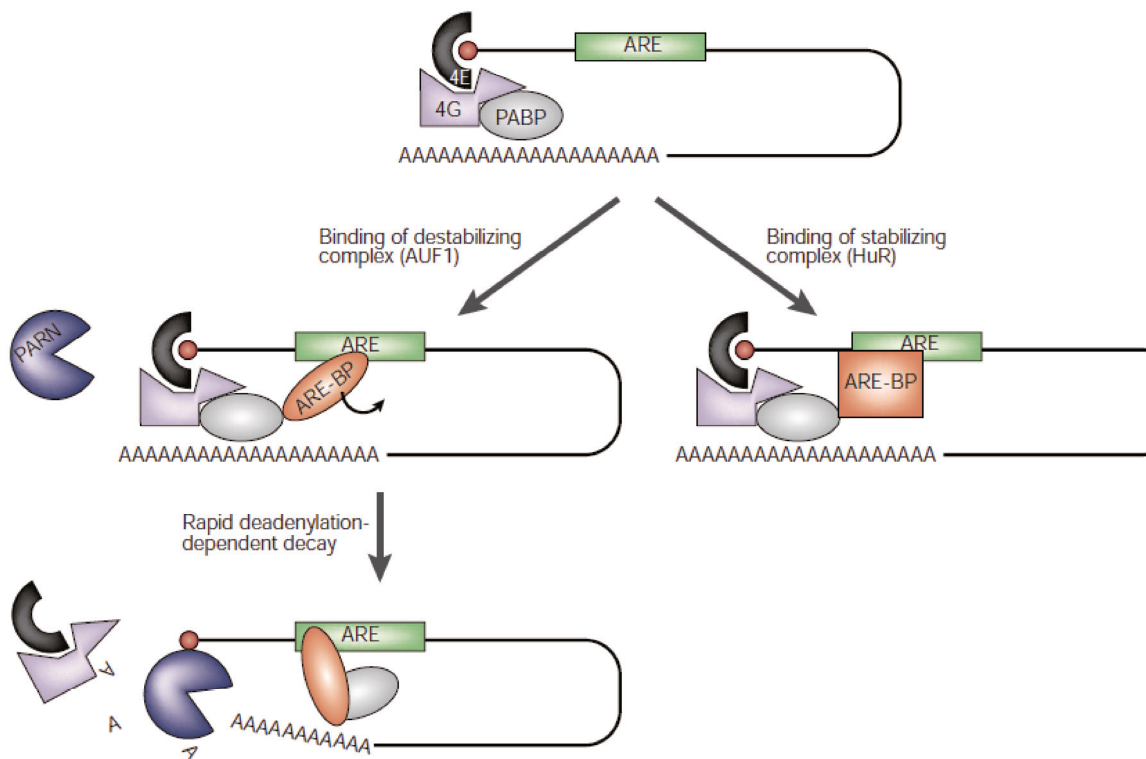

Figure 15: Model of ARE mediated stability of mRNA[169]

III. AIM OF THE THESIS

Human natural killer (NK) cells represent a unique subset of lymphocytes that contribute to the immunosurveillance of tumor-transformed cells. The recognition and elimination of cancer cells by NK cells is tightly regulated by a balance of activating and inhibitory signals mediated through receptor/ligand interactions. The inhibitory killer immunoglobulin-like receptors (KIR) recognize allelic groups of HLA class I molecules on target cells. Engagement of KIRs with ‚self' human leukocyte antigen (HLA) class I molecules inhibits NK cell effector function, whereas absence of these ligands on target cells, a common consequence of tumor transformation, triggers NK cell cytotoxicity. In addition to sensing the lack of ‚self' HLA class I molecules, NK cells need to be stimulated through binding of cell-surface ligands on target cells to specific activating receptors. NKG2D serves as primary activating receptor and interacts with major histocompatibility complex class I (MIC)-related molecules, and UL-16 binding proteins (ULBP). NKG2D ligands (NKG2D-L) are absent or expressed at low levels in healthy tissue, but are frequently overexpressed on the cell surface of malignant cells in response to cellular stress including heat shock, DNA damage and stalled DNA replication. The functional importance of NKG2D and its ligands in cancer immunosurveillance has been demonstrated by accelerated tumor growth in NKG2D-deficient mice and efficient rejection of NKG2D-L+ tumors cells.

Results from clinical hematopoietic stem cell transplantations (HSCT) indicate a curative role for NK cells in some types of human hematopoietic malignancies. Patients with acute myeloid leukemia (AML) undergoing haploidentical HSCT had an improved outcome when the recipient lacked HLA class I ligands for donor inhibitory KIRs. This KIR-HLA class I mismatch provided alloreactivity of NK cells, resulting in an enhanced recognition and elimination of residual malignant cells by the graft-versus-leukemia (GvL) effect. However, the effectiveness of the GvL may be limited since donor-derived NK cells display phenotypical abnormalities and functional immaturity and since the number of potentially alloreactive cells among donor NK cells is highly variable. Adoptive transfer of mature alloreactive NK cells post-transplantation might be a rational approach to increase the GvL effect in order to prevent relapse of AML patients.

Leukemic cells have developed strategies to evade recognition by NK cells. Unlike in solid tumors, where the HLA class I molecules are frequently down-regulated, malignant

leukemic cells express high levels of HLA class I molecules and therefore inhibit the effector function of NK cells through binding to KIR receptors. Furthermore, AML blasts express only low levels of activating NKG2D-L and this prevents NKG2D-mediated activation of NK cells. In consequence, both escape mechanisms decrease the GvL effect of NK cells upon haploidentical HSCT. A better understanding of molecular meachnisms which contribute to regulation of activating and inhibitory ligands in cancer cells may help to develop strategies to increase the recognition and elimination of cancer cells by NK cells.

In the first part of the thesis, our goal was to investigate molecular mechanisms which contribute to the regulation of the activating NKG2D-L, ULBP1. Previous studies have shown that the human NKG2D-L, MICA and MICB, as well as the murine NKG2D-L, MULT-1, are post-transcriptionally regulated by microRNAs and ubiquitination, respectively. The regulation of ULBP1 has not been linked to any post-transcriptional regulation mechanism so far. Since the 2.4kb-long 3'UTR of ULBP1 contains multiple potential regulatory elements, like ARE motifs and microRNA binding sites, our first goal was to investigate, by using luciferase reporter assays, whether the 3'UTR is involved in regulation of ULBP1. The involvement of ARE sites and specific candidate microRNAs in ULBP1 regulation was studied by mutating the regulatory elements in the 3'UTR, by overexpression of candidate microRNAs and by silencing of endogenous microRNAs with antisense molecules.

In the second part of the thesis, our aim was to develop approaches, which may allow to augment recognition and elimination of cancer cells by NK cells. The emphasis of our work was on the NKG2D-L-mediated susceptibility of AML blasts by NK cells. Previous studies have shown that induction of NKG2D-L surface expression can be achieved by treatment of cancer cells with the histone deacetylase inhibitor (HDACi) valproic acid (VA). The first strategy used in our work was to examine the effect of HDACi, VA and trichostatin A, on NKG2D-L expression and its functional consequences. In these experiments, we used both primary human AML blasts or normal human fibroblasts and analyzed the endogenous NKG2D-L expression and the effect of HDACi in ULBP1-3'UTR luciferase reporter assays. A second strategy in our work was to improve the recognition of AML blasts by NK cells, by circumventing the inhibitory effect of KIR signaling. In order to increase alloreactivity of NK cells we generated NK cell lines, expressing only one KIR receptor on the cell surface, and investigated their cytotoxicity towards KIR-HLA class –mismatched primary AML blasts.

Despite their different objectives, both projects have a common aim: to augment the activation of NK cells in order to eliminate cancer cells either by strengthening of the activating signaling or by circumventing the inhibitory signaling. A better understanding, on how activation of NK cells can be increased may contribute to development of new immunotherapeutic approaches in anti-cancer treatment.

IV. RESULTS (PART 1)

1. Post-transcriptional regulation of ULBP1, a ligand for the activating immunoreceptor NKG2D

1.1 Introduction

NKG2D is a major activating immunoreceptor expressed on cytotoxic NK cells and $CD8^{+}$ T cells, playing a key role in tumor surveillance and viral defense[63]. Ligands for NKG2D, ULBPs and MICA/B, are rarely expressed on healthy cells but can be induced upon cellular stress, malignant transformation and viral infection.[104] In consequence, ligand-expressing abnormal cells are recognized by immune effector cells, resulting in triggering of cytotoxicity and cytokine production. However, in many human tumors including acute myeloid leukemia (AML) expression of NKG2D-L is low or absent, contributing to poor recognition by immune effector cells.[37,156] Thus, increasing surface levels of NKG2D-L could represent a therapeutic approach to increase the immunogenicity of tumors. The precise mechanisms of how NKG2D-L are regulated are however incompletely understood. A better understanding of NKG2D-L regulation would be needed in order to be able to modulate the expression of these molecules for therapeutic interventions in cancer treatment.

Discrepancies in mRNA levels and surface expression of NKG2D-L suggest, that not only transcriptional, but also post-transcriptional mechanisms could contribute to the regulation of NKG2D-L.[62] Two important mechanisms of post-transcriptional regulation are microRNA- and ARE-mediated regulation. The regulatory elements, ARE motifs and microRNA binding sites, are mainly located within the 3'UTR of mature mRNAs. Indeed, endogenous microRNAs have been identified to control MICA and MICB protein expression by serving as a threshold mechanism to prevent undesired upregulation of MICA/B resulting from small fluctuations in the amount of MICA and MICB mRNA.[95] Furthermore, miR-520b is induced upon treatment with IFN-γ, and directly regulates MICA by targeting its 3'UTR.[98]

NCBI database search of the NKG2D-L transcripts revealed the presence of 3'UTRs in mRNAs of five out of seven ligands (Fig. 16). ULBP1 and MICB possess long 3'UTRs (2.4kb and 1.4kb, respectively), whereas the 3'UTRs of ULBP2, RAET1E and MICA are clearly shorter (32 – 530 bp). Futhermore, at least one pentameric ARE motif, AUUUA, is present in each 3'UTR, except for RAET1E.

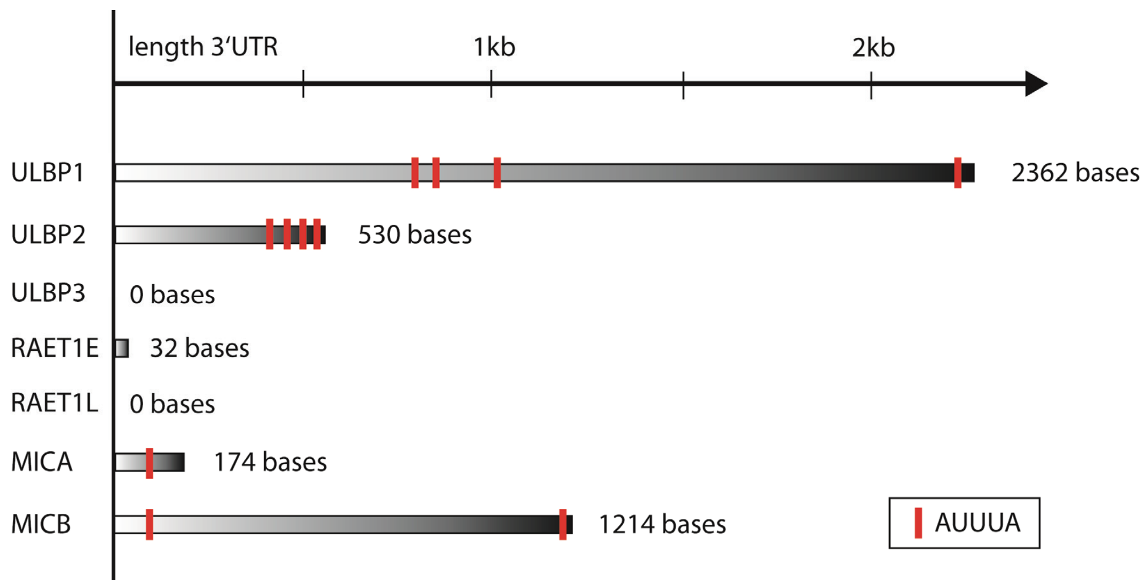

Figure 16: Length of 3'UTRs and presence of ARE motifs

Computational prediction by Targetscan V5.1 algorithm showed, that all NKG2D-L 3'UTRs contain potential microRNA binding sites (Table 7), which are not restricted to a particular region in the 3'UTR but distributed all over the 3'UTR. The vast majority of microRNAs are predicted to target the 3'UTR at only one position, however some microRNAs are potentially capable to bind the 3'UTR at two or more sites (Table 7).

The different properties of NKG2D-L 3'UTRs together with the fact, that the expression level of individual NKG2D-L differs considerably among different tumors and infected cells indicate, that the various NKG2D-L might be regulated by different mechanisms.[63] The presence of four ARE motifs and as well as 195 potential microRNA binding sites in the ULBP1-3'UTR prompted us to investigate if post-transcriptional mechanisms are involved in regulation of ULBP1.

Table 7: Characteristics of NKG2D-L 3'UTRs (March 2010)

	Length of 3'UTR [bp]	Number of ARE sites	prediction by Targetscan V5.1 Number of different miRs	 Total number of miR binding sites in the 3'UTR
ULBP1	2362	4	149	195
ULBP2	530	4	35	35
ULBP3	-	-	-	-
RAET1E	32	-	2	2
RAET1L	-	-	-	-
MICA	174	1	26	28
MICB	1214	2	133	186

1.2 Material and Methods

1.2.1 Cell culture

HeLa (cervical carcinoma cell line), Jurkat (T cell leukemia cell line), K562 (chronic myeloid leukemia cell line) and Raji (Burkitt lymphoma) cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FCS) (Invitrogen) and Penicillin/Streptomycin (Invitrogen). Human dermal foreskin fibroblasts (HFF) were purchased from Invitrogen (Carlsbad, CA) and cultured in Medium 106 and Low Serum Growth Supplement, as provided by the supplier. HFF were treated for 24 hours with the HDAC inhibitor trichostatin A (TSA; Sigma, Buchs, Switzerland), 5-fluorouracil (5-FU; Teva Pharma,Switzerland), aphidicolin (Sigma-Aldrich, St Louis, MO) or valproic acid (VA; Orfiril; Desitin, Liestal, Switzerland) at indicated concentrations.

1.2.2 Cloning of luciferase expression vectors

The full length 3'UTR of ULBP1 was amplified by PCR from I.M.A.G.E. Full Length cDNA clone IRATp970D01103D (imaGenes, Berlin, Germany) with primers containing *Not*I restriction sites (Table 8), subcloned into pGEM-T vectors (Promega, Madison, WI) and inserted into the *Not*I site of the *Renilla* luciferase reporter vector pRL-con (kindly provided by W. Filipowicz, FMI, Basel) immediately downstream of *Renilla* luciferase coding region to produce pRL-U1-UTR (Fig. 17).[245] The orientation of the insert was determined by PCR. Nine fragments of the ULBP1-3'UTR, U1-1 to U1-9, were generated by specific PCR amplification from the ULBP1-3'UTR cDNA clone with fragment-specific forward and reverse primer containing *Not*I sites (Table 8), subcloned into pGEM-T and inserted into the *Not*I site of pRL-con (Fig. 17). The pRL-U1-6dupl construct was generated by amplification of U1-6 from the ULBP1-3'UTR cDNA clone with primers containing *Xba*I sites (Table 8), and inserted into the *Xba*I site of pRL-U1-6, between the *Renilla* luciferase gene and the fragment U1-6.

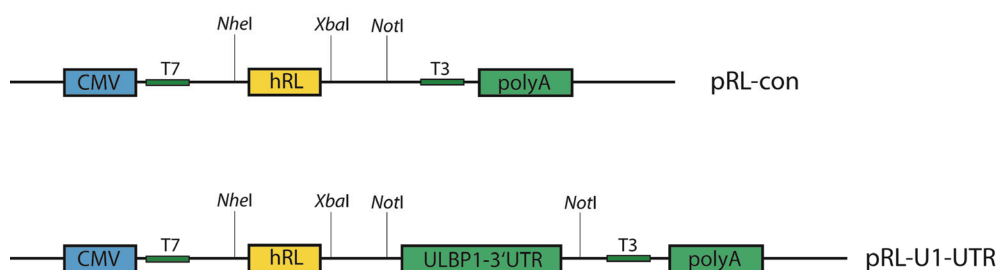

Figure 17: Scheme of pRL-con and pRL-U1-UTR. T7 = T7 promoter; T3 = T3 promoter; CMV = cytomegalovirus; hRL = *Renilla* luciferase.

Mutated fragments of ULBP1-3'UTR, containing base pair substitutions or deletions in potential microRNA seed sequences (Fig. 18) or in predicted ARE sites (Fig. 19), as indicated in the Result sections, were ordered from DNA 2.0 (Menlo Park, CA), subcloned into p-GEM-T vectors and inserted into the *Not*I site of pRL-con. All products were verified by sequencing.

For stable luciferase expression, we used the lentiviral vector MA1 containing two expression cassettes driven by a bidirectional promoter (kindly provided by L. Naldini, Milano).[246] The ΔLNFGR gene was excised by digest with *Xma*I and *Sal*I, and replaced by a *Renilla* luciferase expression cassette. The *Renilla* luciferase gene with and without the full length ULBP1-3'UTR was amplified from pRL-U1-UTR with primers containing *Xma*I or *Sal*I restriction sites (Table 8) and subcloned into pGEM-T. Both fragments were inserted into the lentiviral vector MA1 to produce LV-RL-con and LV-RL-U1-UTR. Lentiviruses were produced as described.[247]

Table 8: Primers for cloning of transient and lentiviral luciferase reporter vectors

vector system	fragment	orientation	sequence
transient luciferase reporter system	full length, U1-1, U1-2, U1-3	for	5' AA GCGGCCGC GGA GAG TTG TTT AGA GTG ACA G 3'
	full length, U1-9	rev	5' AA GCGGCCGC TTC CAT TAT ACA GTG TTT AAT TG 3'
	U1-1	rev	5' AA GCGGCCGC GCC TTT TGC TGG GAT TTC TTG TT 3'
	U1-2	rev	5' AA GCGGCCGC GGC AAC ATA GGA AGA CCC TAC CT 3'
	U1-3	rev	5' AA GCGGCCGC TTA CAG TTT TAG TAC TTA TAT TT 3'
	U1-4	for	5' AA GCGGCCGC AAG AAA TCC CAG CAA AAG GCA TT 3'
	U1-4	rev	5' AA GCGGCCGC GAC CAT CCT GGG CAA CAT AGG AA 3'
	U1-5	for	5' AA GCGGCCGC TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-5	rev	5' AA GCGGCCGC TCC AGG ATT TTT ACA GTT TTA GT 3'
	U1-6	for	5' AA GTCGAC GCGGCCGC TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-6	rev	5' AA GTCGAC GCGGCCGC GGT GTG CAG GTT GAT TTC TGG 3'
	U1-6dupl	for	5' AA TCTAGA TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-6dupl	rev	5' AA TCTAGA GGT GTG CAG GTT GAT TTC TGG 3'
	U1-7	for	5' AA GTCGAC GCGGCCGC CCG CCA TCT AAT CTT CAA TAG 3'
	U1-7	rev	5' AA GTCGAC GCGGCCGC TCC AGG ATT TTT ACA GTT TTA GT 3'
	U1-8	for	5' AA GTCGAC GCGGCCGC TGG AAT ATA ACC TAA GAA ATA CC 3'
	U1-8	rev	5' AA GTCGAC GCGGCCGC TGT AAT GAA CAT ATT GGT GCA GG 3'
	U1-9	for	5' AA GTCGAC GCGGCCGC GTG CTA TTC TCA CCA GCA AG 3'
	U1-9	rev	5' AA GTCGAC GCGGCCGC TTC CAT TAT ACA GTG TTT AAT TG 3'
lentiviral luciferase reporter system	pRL-con, pRL-U1-UTR	for	5' AA TTCCCGGG CTC ACT ATA GGC TAG CCA CC 3'
	pRL-con	rev	5' AA TTGTCGAC CTA GAA TTA CTG CTC GTT CTT C 3'
	pRL -U1-UTR	rev	5' AA TTGTCGAC CAT TAA CCC TCA CTA AAG GGA A 3'

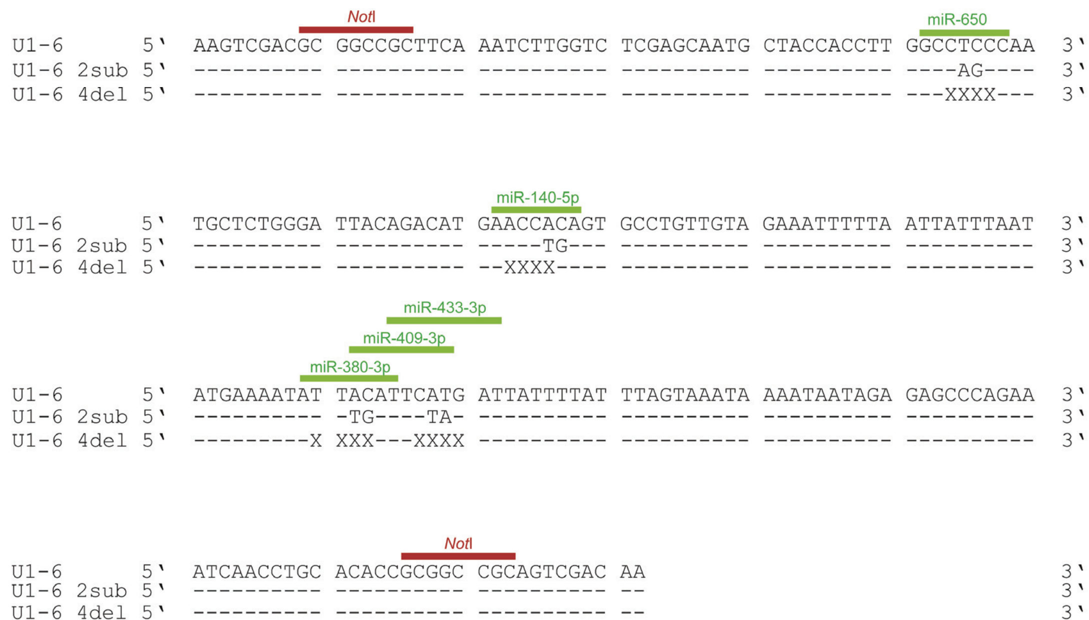

Figure 18: Oligonucleotides with seed sequence mutations in fragment U1-6. The 5' and 3' flanking region contains *Not*I restriction sites. X = deletion

A

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U1-5       5'  AAGTCGACGC GGCCGCTTCA AATCTTGGTC TCGAGCAATG CTACCACCTT GGCCTCCCAA  3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5       5'  TGCTCTGGGA TTACAGACAT GAACCACAGT GCCTGTTGTA GAAATTTTTA ATTATTTAAT  3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---------- -----G----  3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----GGG---  3'

U1-5       5'  ATGAAAATAT TACATTCATG ATTATTTTAT TTAGTAAATA AAATAATAGA GAGCCCAGAA  3'
U1-5 mut   5'  ---------- ---------- -----G---- G--------- ---------- ----------  3'
U1-5 mut2  5'  ---------- ---------- ----GGGG-G GG-------- ---------- ----------  3'

U1-5       5'  ATCAACCTGC ACACCTACCG CCATCTAATC TTCAATAGAA ATGGGCAATG TGGGAAAGAC  3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5       5'  TCCCTATTCG AAAATTAGTG CTGGAATATC TGGCCAACCA TATGCAGAAG AATGAAACTG  3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5       5'  AACCCCTACT TCTCCCCATA TATGTAAAAT AATTCAATAT GGATGAAAGA TTTAAATATA  3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---------- -G--------  3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---------- GGG-------  3'

U1-5       5'  AGTACTAAAA CTGTAAAAAT CCTGGAGCGG CCGCAGTCGA CAA                    3'
U1-5 mut   5'  ---------- ---------- ---------- ---------- ---                    3'
U1-5 mut2  5'  ---------- ---------- ---------- ---------- ---                    3'
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B

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U1-9       5'  AAGTCGACGC GGCCGCGTGC TATTCTCACC AGCAAGGACA GAGAATCAAT CTAAGTGCCC  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  AACAACAGTA AATTCAATGA AAAAAAATGT GGTACATAGA TACGATGGAA AACTATGCAG  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  CCATGAAACA CAAGAAAATC ATGTCCTTTT CAGCAACATG GATGCAACTA GAGGCTATTA  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  TCCTAAGCAA CCTAATGCAA GAACAGAAAA CCACATACTG CATCTTCCCA TTGGAAAGTG  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  GCAGCTAAAC ATTAAATTCG CATGAACCAC AGATGCTGGA GATCACCAGA CCGGGGAGAG  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  AAGAGGGGCA CCTGGGCTGA AAAACACACC TGTTGGGTAT CATGCTTACT GTCTGGGCGA  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  TGGGATCATT GGGACACCAA GCCTCAGCTT CTCAAATTCT ACCCATGTAA CAAACCTGTA  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  TATGTACCTT GTATTATATA GGTTGAAATT AAAGATGAAT AAATAAAATA AAATGACACA  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  AGGCCAAAAA CAAATGGGTT TAACTGACCA GAGCGAGAG AACTCTGCACT ATGAACCCAA  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  ACCCAGCTCA AAAAGATAAA ATCTAGTCAT TTAAGATAAT CATAAGTTGT ATGATGATAA  3'
U1-9 mut2  5'  ---------- ---------- ---------G GG-------- ---------- ----------  3'

U1-9       5'  TTGTATAAAA ATTTGTATGA TGATAATTGT ATAATAATTA TACATGAAAG TGCCAAAACC  3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9       5'  CTACAATTAA ACACTGTATA ATGGAAGCGG CCGCAGTCGA CAA                    3'
U1-9 mut2  5'  ---------- ---------- ---------- ---------- ---                    3'
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Figure 19: Mutations in ARE sites of fragment (A) U1-6 and (B) U1-9. Red letter indicate *Not*I restriction sites.

1.2.3 Luciferase assay

Jurkat cells ($6x10^5$ cells/well) were transfected in duplicates in 24-well plates with 20ng of luciferase reporter vectors and 250ng of the vector pGL4.13 containing *Firefly* luciferase (Promega), using Lipofectamine 2000 (Invitrogen). HeLa cells were plated at $4x10^4$ cells/well one day prior to transfection with 20ng of luciferase reporter vector and 150ng of pGL4.13. *Renilla* and *Firefly* activities were measured 48 hours after transfection using the Dual-Luciferase Reporter Assay (Promega) on a MicroLumat Plus reader (Berthold Technologies, Bad Wildbad, Germany). *Renilla* luciferase light values were divided by *Firefly* luciferase light values, and ratios were normalized against control plasmid pRL-con. In cells stably transduced with LV-RL-con or LV-RL-U1-UTR, *Renilla* luciferase activity was measured with the *Renilla* Luciferase Assay (Promega) using equal cell numbers and normalized to the percentage of GFP expressing cells. Paired Student's t-test was used to compare the effect of different reporter vectors on luciferase activity.
For microRNA silencing, HeLa and Jurkat cells were co-transfected with 100 nM of anti-miR-140-5p (Ambion, Austin, TX) and luciferase reporter constructs, using Lipofectamine 2000. Luciferase activity was measured 48 hours after transfection.

1.2.4 Flow cytometry

NKG2D-L surface expression was determined using multiparametric FACS (FACSCalibur; BD Biosciences). Cells were stained with control mouse IgG1 (BD Biosciences) or unconjugated mouse monoclonal antibodies (mAbs) against ULBP1 (M295; kind gift of D. Cosman, Amgen, Seattle, WA), MICA/B and HLA class I (BD Biosciences) at 10 µg/mL and with secondary goat-anti mouse IgG-FITC (Jackson ImmunoResearch, West Grove, PA) or goat-anti mouse IgG-Alexa647 (Molecular Probes, Invitrogen). For staining of HFF, the directly labeled mouse mAb ULBP1-PE (R&D Systems, Minneapolis, MN) or mouse IgG2a-PE isotype (BD Biosciences) was used. Propidium iodide (Sigma) was used to exclude dead cells from analysis. Cells were acquired using FACS (FACSCalibur; BD Biosciences) and analysis was performed using FlowJo software (Tree Star, Ashland OR). Surface expression levels of NKG2D-L were defined as the mean fluorescence intensity ratio (MFIR) of values obtained with specific mAbs divided by values given by secondary or control mAbs.[35]

1.2.5 Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicates using *Power* SYBR Green PCR master mix on an ABI Prism 7000 (Applied Biosystems) under the previously described conditions.[248] The primer pairs used for quantification of specific mRNAs are listed in Table 9 and were in part described previously. PCR reactions were normalized to RPL19 for Drosha, ULBP1, MICA and MICB transcripts using the $2^{-\Delta\Delta CT}$ method.[248]

To quantify luciferase transcripts after transient transfection of reporter vectors, total RNA was treated with TURBO DNA-free Kit (Ambion, Austin, TX) and cDNA was generated as described above. Control reactions without reverse transcriptase were performed. Primers specific for *Renilla* and Firefly luciferase are listed in Table 9. *Renilla* luciferase transcripts were normalized to *Firefly* luciferase transcripts.

Table 9: Primers for real-time qPCR. * published in[249]; ** published in[67].

gene		sequence
ULBP1 *	for	5' GTA CTG GGA ACA AAT GCT GGA T 3'
	rev	5' AAC TCT CCT CAT CTG CCA GCT 3'
MICA **	for	5' ACA ATG CCC CAG TCC TCC AGA 3'
	rev	5' ATT TTA GAT ATC GCC GTA GTT CCT 3'
MICB **	for	5' TGA GCC CCA CAG TCT TCG TTA C 3'
	rev	5' TGC CCT GCG TTT CTG CCT GTC ATA 3'
Drosha	for	5' GCA GTT ATT TGG ACG CTT GC 3'
	rev	5' AGT TGT CGA TCA GTA TTT GGC 3'
RPL19	for	5' GAT GCC GGA AAA ACA CCT TG 3'
	rev	5' TGG CTG TAC CCT TCC GCT T 3'
Renilla luciferase	for	5' GAC AAG ATC AAG GCC ATC GTC 3'
	rev	5' CTC GAT CAC GTC CAC GAC AC 3'
Firefly luciferase	for	5' GGG CTG AAT ACA AAC CAT CG 3'
	rev	5' GTT GTA GAT GTC GTT AGC TGG 3'

Stem-loop quantitative RT-PCR for mature microRNAs (TaqMan microRNA Assays, Applied Biosystems) was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) according manufacturer's recommendation. Synthetic microRNAs miR-16, miR-140-5p, miR-496 and miR632 (kindly provided by I. Beuvink, Novartis, Basel, Switzerland)

were used as positive control. PCR reactions were run in duplicates, and microRNA expression, relative to hsa-miR-16, was calculated.[248] The threshold of microRNA expression was defined as $\Delta CT(\text{miR-16} - \text{miR-X}) \leq 12$.

1.2.6 MicroRNA overexpression

Complementary oligonucleotides designed to form artificial pre-microRNA hairpins (Table 10; ordered from Eurogentec, Seraing, Belgium), were annealed by heating to 95°C for 5 minutes and slow cooling to room temperature, phosphorylated by using T4 polynucleotide kinase and inserted into the *Hind*III and *Bgl*II sites of a modified pSUPER vector.[95,250] The overexpression vectors were transiently transfected into Jurkat cells with indicated concentrations, using Lipofectamine 2000, and FACS, luciferase assay and isolation of RNA was performed 48 hours later.

Table 10: Oligonucleotides for cloning of microRNA-overexpression vectors

microRNA	Oligo	Sequence
miR-140-5p	Oligo1	5' GATCCCC**AGTGGTTTTACCCTATGGTAG**TTCAAGAGACTACCATAGGGTAAAACCACTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAAGTGGTTTTACCCTATGGTAGTCTCTTGAACTACCATAGGGTAAAACCACTGGG 3'
miR-380-3p	Oligo1	5' GATCCCC**TATGTAATATGGTCCACATCTT**TTCAAGAGAAAGATGTGGACCATATTACATATTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAATATGTAATATGGTCCACATCTTTCTCTTGAAAAGATGTGGACCATATTACATAGGG 3'
miR-381	Oligo1	5' GATCCCC**TATACAAGGGCAAGCTCTCTGT**TTCAAGAGAACAGAGAGCTTGCCCTTGTATATTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAATATACAAGGGCAAGCTCTCTGTTCTCTTGAAACAGAGAGCTTGCCCTTGTATAGGG 3'
miR-409-3p	Oligo1	5' GATCCCC**CGAATGTTGCTCGGTGAACCCCT**TTCAAGAGAAGGGGTTCACCGAGCAACATTCGTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAACGAATGTTGCTCGGTGAACCCCTTCTCTTGAAAGGGGTTCACCGAGCAACATTCGGGG 3'
miR-433-3p	Oligo1	5' GATCCCC**ATCATGATGGGCTCCTCGGTGT**TTCAAGAGAACACCGAGGAGCCCATCATGATTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAATCATGATGGGCTCCTCGGTGTTCTCTTGAAACACCGAGGAGCCCATCATGATGGG 3'
miR-578	Oligo1	5' GATCCCC**CTTCTTGTGCTCTAGGATTGT**TTCAAGAGAACAATCCTAGAGCACAAGAAGTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAACTTCTTGTGCTCTAGGATTGTTCTCTTGAAACAATCCTAGAGCACAAGAAGGGG 3'
miR-650	Oligo1	5' GATCCCC**AGGAGGCAGCGCTCTCAGGAC**TTCAAGAGAGTCCTGAGAGCGCTGCCTCCTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAAGGAGGCAGCGCTCTCAGGACTCTCTTGAAGTCCTGAGAGCGCTGCCTCCTGGG 3'

1.2.7 ULBP1 overexpression

HeLa and Jurkat cells were transfected with 200ng or 400ng of the ULBP1 overexpression vector RSV.5ULBP1 or the control vector RSV.5neo[251] (kindly provided by A. Steinle, Frankfurt a. Main, Germany), using Lipofectamine 2000. Surface expression levels of ULBP1 were measured 48 hours after transfection.

1.2.8 Knockdown of Drosha with short hairpin RNA (shRNA)

Lentiviral vectors containing a Drosha-specific shRNA (LV-shDrosha) or a control shRNA (LV-shControl), as well as a GFP expression cassette were kindly provided by O. Mandelboim, Jersualem, Israel.[95] Lentiviruses were produced as described and HeLa cells were transduced.[247] The number of GFP^+ cells was measured by FACS to assess transduction efficiency. NKG2D-L surface expression and RNA levels were measured five days after infection. To perform the luciferase assay, HeLa cells were transduced with pRL-con or pRL-U1-UTR, sorted for GFP and subsequently transduced with LV-shDrosha or LV-shControl. Seven days upon the second infection, luciferase activity was measured as described above.
The doxycycline (DOX)-inducible lentivector shDrosha and the control vector pTIG were kindly provided by J. Rossi, Duarte, CA. [252] Lentiviruses were produced as described and HeLa cells were transduced.[247] The number of GFP^+ cells was measured by FACS to assess transduction efficiency. Infected cells were treated for eight days with DOX (1 µg/mL) (Clontech, Mountain View, CA) and NKG2D-L surface expression and RNA levels were measured.

1.3 Results

1.3.1 Role of the 3'UTR in regulation of ULBP1 expression

Since various cells and tissues were shown to express ULBP1 mRNA in the absence of ULBP1 protein detectable on the cell surface,[60] these discrepancies implicate that post-transcriptional mechanisms might be involved in regulation of ULBP1 expression. To investigate whether the ULBP1-3'UTR is involved in regulation of ULBP1 gene expression, we constructed a *Renilla* luciferase reporter plasmid containing the 2.4 kb full length 3'UTR of ULBP1 (pRL-U1-UTR; Fig. 20A) and transiently transfected the construct into tumor cell lines. The transfection efficiency was assessed by co-transfection with a *Firefly* luciferase plasmid. Upon transfection of pRL-U1-UTR into HeLa, Jurkat and K562 cells, luciferase activity was reduced to13.1%±2.5%, 7.3%±0.8% and 25.7%±9.8% compared to control luciferase vector without 3'UTR (pRL-con) (Fig. 20A).

In order to examine the role of the ULBP1-3'UTR in primary cells and cell lines, which are resistant to transient transfection with lipofectamine, we additionally generated a lentiviral luciferase vector containing the ULBP1-3'UTR (LV-RL-U1-UTR; Fig. 20B). Transduction efficiency was monitored by measuring the number of GFP^{+} cells by FACS. In accordance with the transient reporter system, luciferase activity was strongly reduced in Jurkat, HeLa and K562 cells upon stable transduction with LV-RL-U1-UTR compared to control construct LV-RL-con (Fig. 20B). Furthermore, the luciferase activity in Raji cells and primary human foreskin fibroblasts (HFF) was reduced to 12.0%±0.8% and 38.3%±3.5% upon transduction with LV-RL-U1-UTR (Fig. 20B). These data indicate, that the ULBP1-3'UTR is involved in regulation of ULBP1 expression.

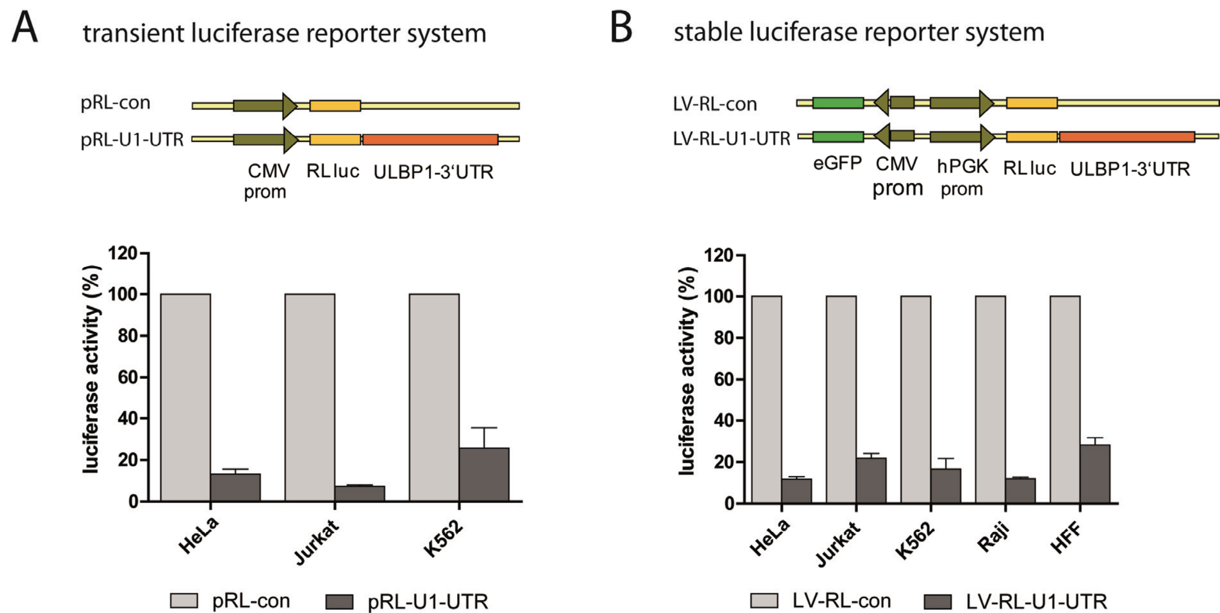

Figure 20: Scheme of luciferase reporter constructs and luciferase activity in tumor cell lines and primary human foreskin fibroblasts. (A) The 2.4 kb full length 3'UTR of ULBP1 was inserted downstream of a luciferase open reading frame into the parental vector pRL-con, resulting in pRL-U1-UTR. Luciferase activity of pRL-con (light grey) and pRL-U1-UTR (dark grey). Results are means ± SEM of duplicates (Hela: n=14; Jurkat: n=20; K562: n=2). (B) The full length ULBP1-3'UTR, fused to the luciferase gene or the luciferase gene alone were inserted into a lentiviral vector (LV-RL-con and LV-RL-U1-UTR, respectively). Luciferase activity was measured seven days after transduction with LV-RL-con (light grey) and LV-Rl-U1-UTR (dark grey). Results are means ± SEM (Hela: n=8; Jurkat: n=5; K562: n=2; Raji: n=2; HFF: n=4).

1.3.2 Role of ARE in regulation of ULBP1 expression

Sequence analysis revealed the presence of four AUUUA motifs in the 3'UTR of ULBP1 mRNA (see Fig. 16). ARE motifs within the 3'UTR are crucial determinants of transcript instability in mammalian cells.[242] To determine the contribution of ARE to regulation of ULBP1 expression, we simultaneously mutated three AUUUA sites within a 367bp-long fragment (U1-5) of the ULBP1-3'UTR (Fig. 19A and Fig. 21A) by substitution of one or three nucleotides, giving rise to pRL-U1-5-AREmut1 (ATTTA to ATGTA) and pRL-U1-5-AREmut2 (ATTTA to AGGGA). In addition, a ARE-like site ATTTTA located in fragment U1-5 was mutated (ATTTTA to ATGTTA or AGGGGA). Likewise, we inactivated one AUUUA motif within a 669bp-long fragment (U1-9) by replacement of three nucleotides (ATTTA to AGGGA; see Fig. 19B and Fig. 21A), resulting in luciferase reporter vector pRL-

U1-9-AREmut2. Transfection of the mutated reporter plasmids into HeLa and Jurkat cells did not result in an increased luciferase activity compared to the unmutated constructs (Fig. 21B) as would be expected from inactivation of mRNA destabilizing elements. Instead, a reduction in luciferase activity was observed in HeLa cells (U1-5: 100% vs. 60.5%±6.64% (p=0.0040) vs. 87.3%±2.1%; U1-9: 100% vs. 77.6%±3.2%), and similar results were obtained for Jurkat cells. Therefore we conclude, that ARE sites are unlikely to play a major role in negative regulation of ULBP1.

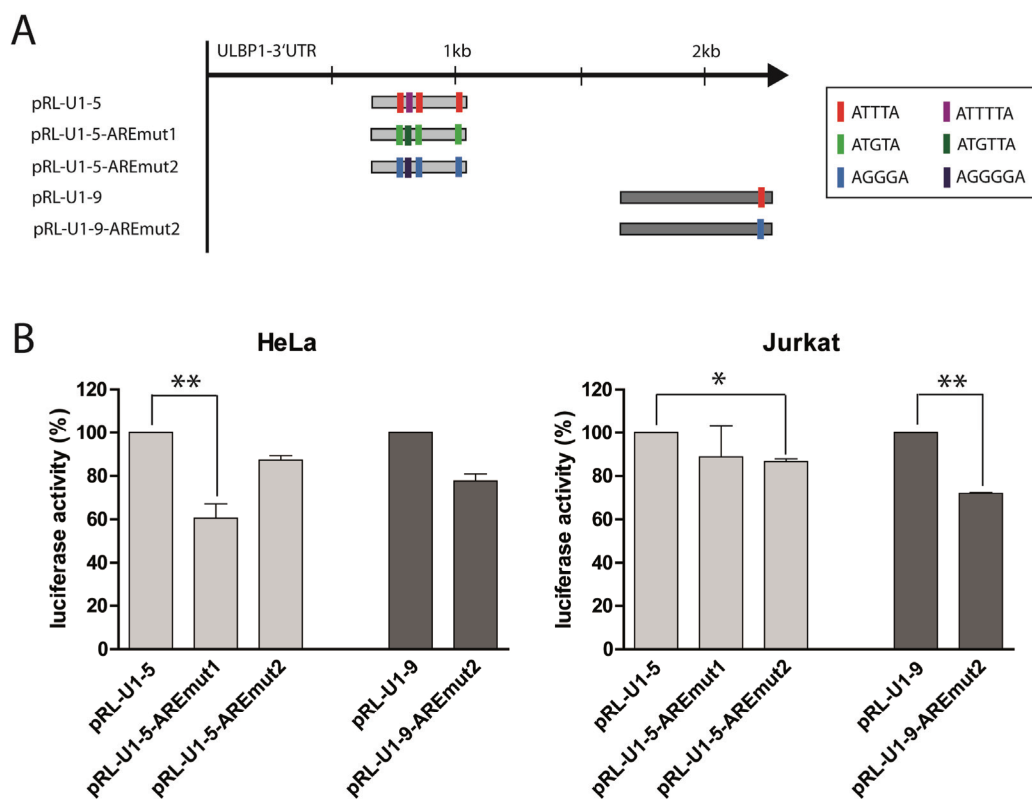

Figure 21: Mutation of ARE in two regions of the U1-3'UTR. (A) Schematic localization of ARE in fragment U1-5 and U1-9 of the U1-3'UTR. The sequence motifs ATTTA (red) and ATTTTA (purple) were mutated by single base substitutions T to G (light and dark green) or by substitution of 3 bases TTT to GGG (light blue) and 4 bases TTTT to GGGG (dark blue), respectively. Mutated fragments were inserted into pRL-con. (B) Luciferase activity was measured upon transient transfection of wild-type and mutated pRL-U1-5 (light grey) and pRL-U1-9 (dark grey) into HeLa and Jurkat cells and normalized to the activity of the corresponding wild-type reporter vectors (pRL-U1-5 and pRL-U1-9, respectively). Results are means ± SEM of duplicates (n=2-5 for HeLa and Jurkat). * = $p < 0.05$; ** = $p < 0.01$.

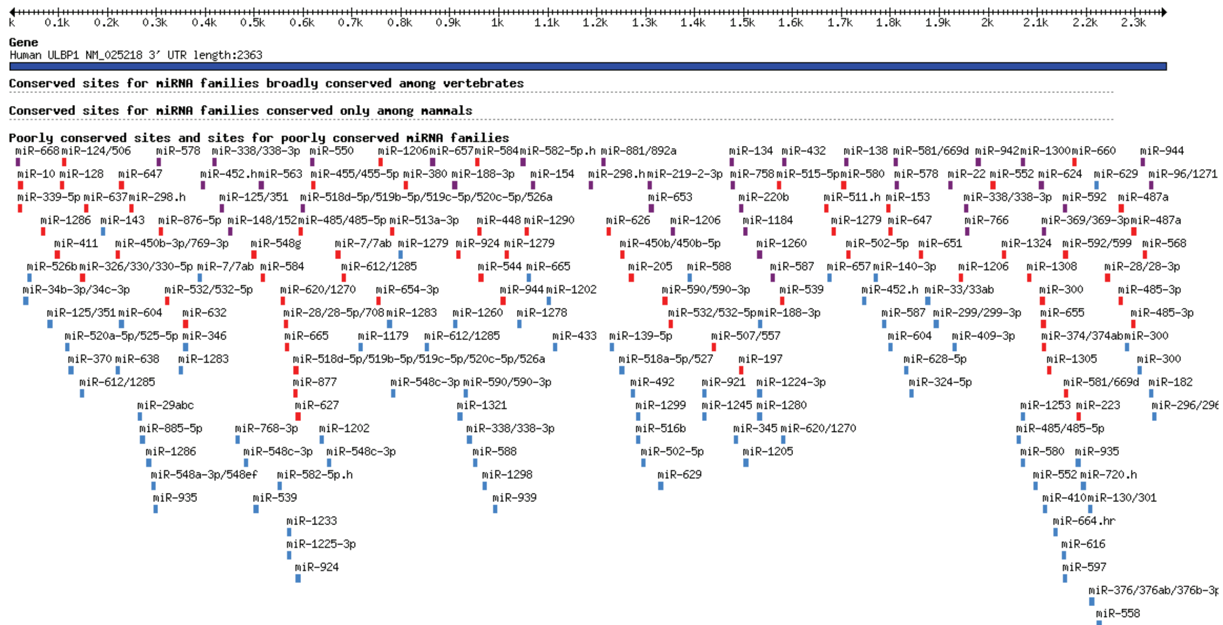

Figure 22: Prediction of potential microRNA binding sites in the ULBP1-3'UTR. Prediction was performed using Targetscan V5.1 (March 2010)

1.3.3 Role of ULBP1-3'UTR fragments in regulation of ULBP1 expression

In order to examine the involvement of microRNAs in regulation of ULBP1 expression, we searched for potential microRNA binding sites in the ULBP1-3'UTR by computational prediction. Analysis by the Targetscan algorithm revealed, that potential regulatory elements are distributed over the entire ULBP1-3'UTR (Fig. 22). To identify regions with high regulatory potential, we cloned nine fragments of the ULBP1-3'UTR (length: 178 - 1052 bp) downstream of the luciferase gene and transiently expressed the constructs pRL-U1-1 to pRL-U1-9 in Jurkat and HeLa cells (Fig. 23A). Significant reduction of luciferase activity compared to pRL-con was observed for all fragments (Fig. 23B). Luciferase activity ranged from 19.1%±2.0% (U1-3) to 49.2%±8.4% (U1-7) in HeLa, and 23.8%±5.2% (U1-3) to 61.9%±14.6% (U1-1) in Jurkat cells. Remarkably, none of the fragments was as potent as the the full length reporter construct pRL-U1-UTR. These observations suggest, that regulatory elements are located within the whole 3'UTR rather then restricted to specific areas.

Luciferase activity was most efficiently reduced in cells transfected with pRL-U1-3 (HeLa: 19.1%±2.0%; Jurkat: 23.8%±5.2%). Among the fragments within the region U1-3, less pronounced reduction of luciferase activity was observed for fragment U1-1 (61.9%±14.6%), U1-4 (56.6%±18.5%), and U1-5 (36.5%±3.6%) in Jurkat cells. The luciferase activity

observed for the subfragment U1-6 was only slightly higher compared to U1-5 (41.1%±1.5% vs. 36.9%±3.6%) in Jurkat cells, and almost equal (39.6%±3.9% vs. 40.0%±3.4%) in HeLa cells. Therefore we decided to concentrate in our further studies on the 178-bp-long fragment U1-6, which contains only a limited amount of binding sites for candidate microRNAs. Moreover, we included fragment U1-9 in our studies, since luciferase activity upon transfection with pRL-U1-9 revealed a highly reduced lucferase activitiy (25.6%±1.4%) in Jurkat cells, which was comparable to luciferase activity upon transfection with pRL-U1-3.

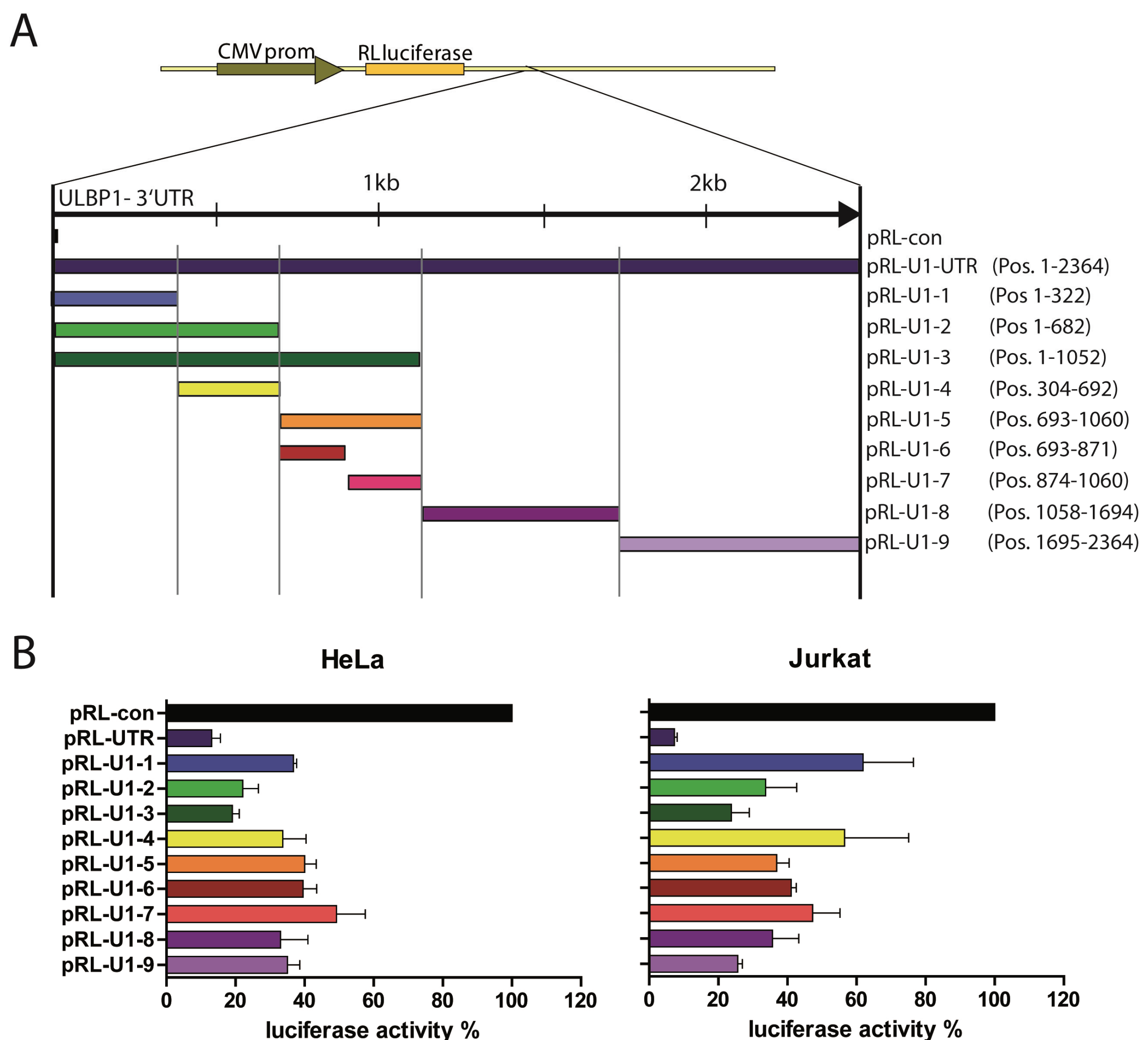

Figure 23: Luciferase activity of reporter plasmids, containing fragments of U1-3'UTR. (A) Fragments of the full length ULBP1-3'UTR were inserted downstream of the Renilla luciferase gene into the parental vector pRL-con. (B) Luciferase activity was measured upon transient transfection into HeLa and Jurkat cells and normalized to pRL-con. Results are means ± SEM of duplicates (n=2-14 for HeLa and n=2-20 for Jurkat). prom = promoter; RL = *Renilla*.

Binding of microRNAs to the 3'UTR of target genes results in translation repression or mRNA degradation.[184] To investigate the post-transcriptional mechanism leading to reduced luciferase activity, we measure reporter mRNA levels upon transient transfection with luciferase constructs (full-length ULBP1-3'UTR and fragments) in HeLa and Jurkat cells by qPCR, using primers located in the *Renilla* luciferase. mRNA levels of *Renilla* luciferase were first normalized to mRNA levels of *Firefly* luciferase gene and then to pRL-con. mRNA levels of different reporter constructs were subsequently correlated with luciferase activity (Fig. 24). Independent of the transfected fragments, levels of reporter mRNA in HeLa cells were low (2.4% to 21.8%) compared to control vector, indicating that reduced luciferase activity is most likely caused by degradation of mRNA. In contrast, high levels of reporter mRNA, ranging from 30.8%±2.4% to 110.4%±19.7%, were detected in Jurkat cells which is best explained by translational repression rather then RNA degradation. This experiments suggests, that the expression of ULBP1 in HeLa and Jurkat cells might be regulated by two different mechanisms.

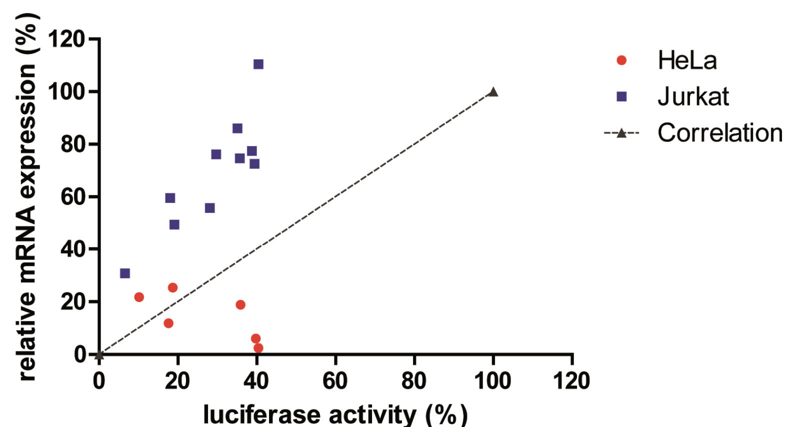

Figure 24: Levels of reporter mRNAs correlated with luciferase activity in Hela and Jurkat cells. mRNA expression levels of luciferase reporters and luciferase activity were measured upon transient transfection of reporter constructs into HeLa (●) and Jurkat (■) cells. (n=1-4)

1.3.4 Role of region U1-6 in regulation of ULBP1

Since target site multiplicity is thought to enhance the degree of translational repression[184], we generated a reporter vector containing a duplication of fragment U1-6 (pRL-U1-6dupl). Compared to pRL-U1-6, luciferase activity was reduced in HeLa (100% vs. 81.3%±3.9%) and Jurkat (100% vs. 71.6%±2.8%) cells transfected with pRL-U1-6dupl (Fig.25). These results provide additional evidence, that fragment U1-6 contains regulatory elements of ULBP1.

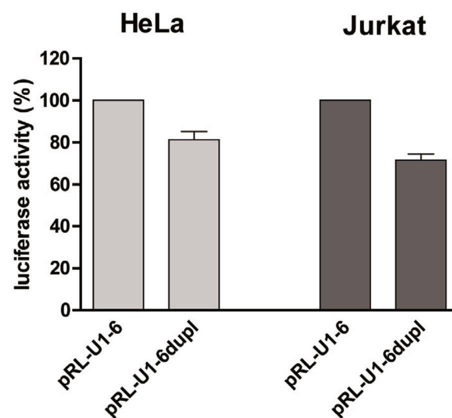

Figure 25: Luciferase activity of reporter constructs containing a duplication of U1-6. Luciferase activity was measured upon transient transfection into HeLa and Jurkat cells and normalized to pRL-con. Results are means ± SEM of duplicates. (n=2)

1.3.5 Role of specific microRNAs in regulation of region U1-6 and U1-9

Computational analysis

In an attempt to identify microRNAs that target ULBP1 mRNA, we used computer algorithms to search for microRNA binding sites in the ULBP1-3'UTR. The accuracy of programs to predict microRNA binding sites in ULBP1-3'UTR is limited, because ULBP1 does not show sequence conservation which is one of the criteria that most of these programs are based upon. We used TargetScan V4.2 and 5.1 to predict candidate microRNAs and combined the results with predictions obtained from three alternative computational methods (DIANA microT v3.0, ElMMO v3 and Micro-Inspector v1.5). Since multiplicity of target sites is not

necessarily required, but thought to be an important factor for efficient translational inhibition, we included this criterion in our selection process.[184]

Based on the strong suppressing effect on luciferase activity, we focused on region U1-6 in a first round of candidate selection. From 7 and 11 microRNAs, predicted with TargetScan V4.2 and V5.1 to target region U1-6, we selected 6 microRNAs for further investigation (Table 11). miR380-3p, miR-548c, miR-612 and miR-657 were predicted to target U1-6 by both algorithms, V4.2 and V5.1. Furthermore, we selected miR-140-5p, since this microRNA is, according to V4.2, supposed to target U1-6, U1-9 as well as another region in the 3'UTR. miR-409-3p was predicted to target U1-6 (V4.2) or U1-9 (V5.1). Since miR-1179, miR-1206, miR-1279, miR-1283 and miR1285 were discovered only recently, we excluded these particular miRs. Furthermore, we excluded miR-513-3p and miR-654-3p since they were predicted by V5.2 to target the 3'UTR only at one site.

Table 11: First round of candidate microRNA selection. Targetscan V4.2 and V5.1 were used for prediction of candidate microRNAs in fragment U1-6. Selected microRNAs are indicated in red. miR801h was identified as a fragment of U11 splisosomal RNA and therefore removed from miRBase (www.mirbase.org)

	prediction algorithm						
	Targetscan V4.2			Targetscan V5.1			
microRNA	predicted to target U1-6	predicted to target U1-9	no. of predicted binding sites in full length 3'UTR	predicted to target U1-6	predicted to target U1-9	no. of predicted binding sites in full length 3'UTR	selected for further examination
miR-140-5p	X	X	3				X
miR-380-3p	X		1	X		1	X
miR-409-3p	X		1		X	1	X
miR-513-3p				X		1	
miR-548c	X		3	X		3	X
miR-612	X		3	X		3	X
miR-654-3p				X		1	
miR-657	X		2	X		2	X
miR-801h	(X)		(1)				
miR-1179				X		1	
miR-1206				X	X	3	
miR-1279				X		3	
miR-1283				X		2	
miR -1285				X		3	

In a second round of candidate selection, we chose 7 additional microRNAs (miR-338-3p, miR-381, miR-433-3p, miR-496, miR-576, miR-632, miR-650) for further studies (Table 12). All of these microRNAs were predicted to target region U1-6 or U1-9 at least once. Except

for miR-496 and miR-650, all candidate microRNAs were predicted with at least three different algorithms to target the ULBP1-3'UTR. Furthermore, all candidate microRNAs, except miR-650, were predicted to target the ULBP1-3'UTR at least at two independent binding sites. The positions and numbers of potential microRNA binding sites for all selected microRNAs are indicated in Figure 26.

Table 12: Second round of candidate microRNA selection. TS = Targetscan; E = Elmmo v3, M = Micro-Inspector v1.5, D = Diana microT v3.0

microRNA	prediction algorithm	predicted to target U1-6	predicted to target U1-9	total number of binding sites in the full length 3'UTR
miR-338-3p	TS V4.2, TS V5.1, E, M, D		X	3
miR-381	TS V4.2, E, D		X	3
miR-433-3p	TS V4.2, TS V5.1, E	X		3
miR-496	TS V4.2		X	2
miR-578	TS V4.2, TS V5.1, E, M, D		X	5
miR-632	TS V4.2, TS V5.1, E, M, D	X		5
miR-650	M	X		1

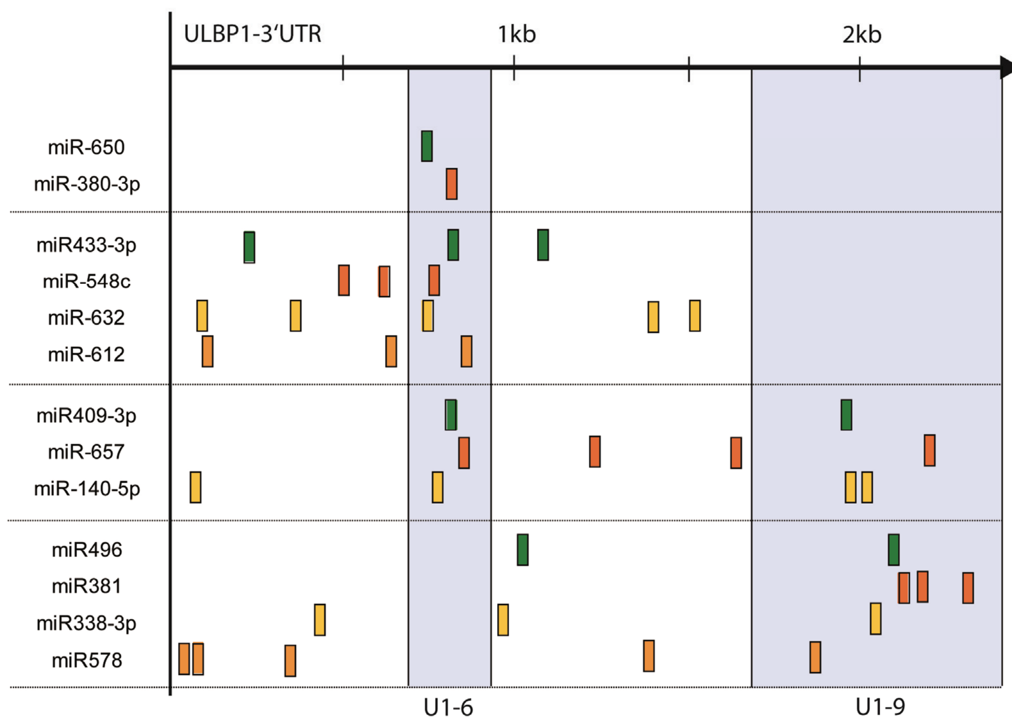

Figure 26: Localization of potential microRNA binding sites in the ULBP1-3'UTR.

Expression of candidate microRNAs

A candidate microRNA responsible for reduced luciferase activity upon transfection with reporter constructs would need to be expressed in Jurkat, HeLa and HFF. To assess the microRNA expression level of candidate microRNAs, we performed qPCR using microRNA specific Taqman primers. Starting with 0.44 ng RNA per PCR reaction, miR-16 level was equally high expressed in all cell lines examined with an average cycle number (C_T) of 22.9±0.8 (range from 21.8±0.3 to 24.3±0.4 C_Ts)(Fig. 27). This observation is in accordance with published data.[253-254] Therefore, miR-16 was used as housekeeping microRNA to normalize qPCR results.

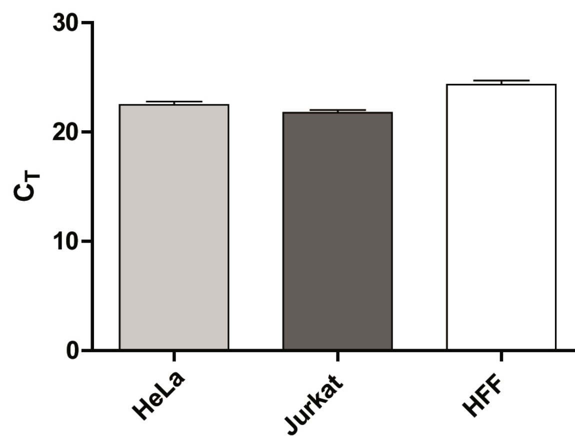

Figure 27: Expression of miR-16 in HeLa Jurkat and HFF. Results are means ± SEM of absolute cycle numbers (C_T), measured in duplicates. (n=6-8)

Of the 13 candidate microRNAs, 6 microRNAs (miR-140-5p, miR-381, miR-409-3p, miR-433-3p, miR-496, miR650) were expressed in HFF, miR-140-5p and miR-650 were expressed in Jurkat cells and miR-140-5p, miR-409-3p, miR-433-3p and miR-650 were expressed in HeLa cells (Fig. 28). All other microRNAs were either not detectable (C_T < 37) or only weakly expressed below the threshold, which was defined as $2^{-\Delta CT(\text{miR-16} - \text{miR-X})} \leq 2^{-12}$. These data are in accordance with the literature, reporting a global decrease of microRNAs in many tumors.[193]

Since many of the microRNA were undetectable in all three cell lines tested, we used synthetic microRNA as a positive control for four TaqMan microRNA assays (miR-16, miR-140-5p, miR-496, and miR-632). Positive signals were detected with all four assays (data not shown), therefore excluding technical problems in our measurements.

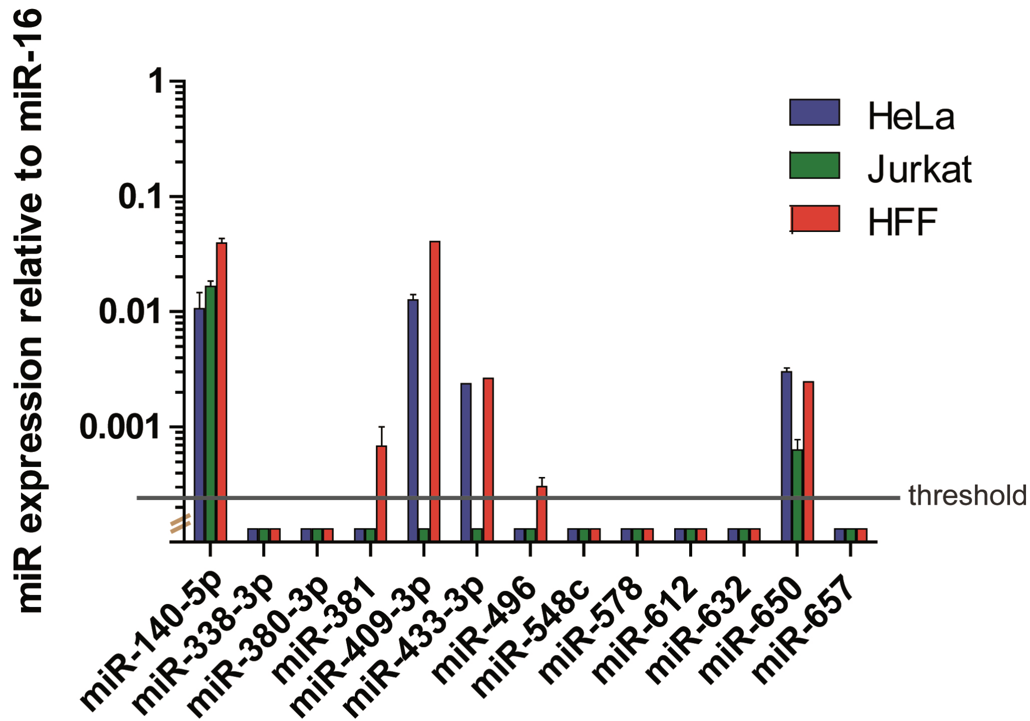

Figure 28: Endogenous expression of candidate microRNAs in HeLa, Jurkat and HFF. Results are means ± SEM of duplicates (n=1-5).

Mutation of microRNA seed sequences

To determine, if the microRNAs expressed in HeLa and Jurkat are involved in targeting the ULBP1-3'UTR, we generated two mutants (Fig. 18 and 29A) of fragment U1-6 by introducing base pair substitutions (U1-6sub) or base pair deletions (U1-6del) to simultaneously disrupt the seed sequence of five microRNA binding sites (miR-140-5p/380-3p/409-3p/433-3p/650). In comparison to the unmutated construct pRL-U1-6 (Fig. 29B), luciferase activity was significantly higher in Jurkat cells (100 vs 118.7%±0.5% (p = 0.0157) vs. 111.3%±3.6%) and slightly higher in HeLa cells (100% vs. 116.7%±12.0% vs. 114.9%±8.0%). These results suggest that at least some of the candidate microRNAs expressed in HeLa and Jurkat cells may contribute to regulation of ULBP1 within the region U1-6 of 3'UTR.

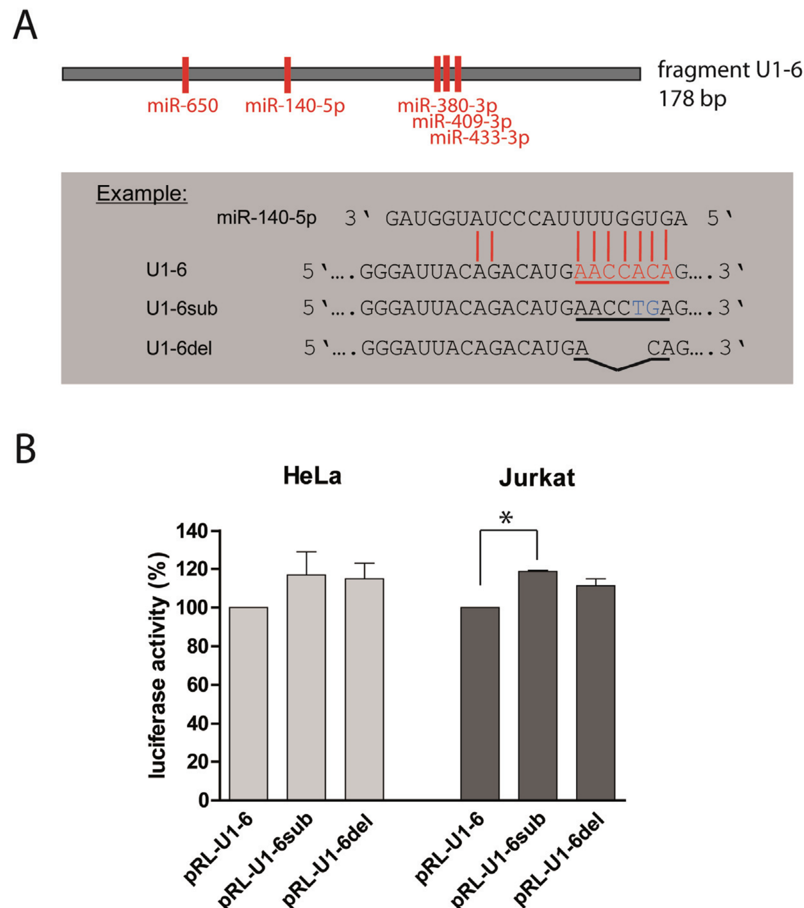

Figure 29: Effect of seed sequence mutations on luciferase activity. (A) Mutations of five seed sequences were simultaneously introduced into the fragment U1-6. The seed sequences were mutated by substitution of two bases (U1-6sub) or deletion of four bases (U1-6del). (B) Luciferase activity was measured upon transient transfection of luciferase vectors, containing seed sequence mutations in fragment U1-6 (pRL-U1-6 2sub and pRL-U1-6 4del, respectively), into HeLa and Jurkat cells. Luciferase activity was normalized to the activity of the non-mutated pRL-U1-6 vector. Results are means ± SEM of duplicates (n=2 for HeLa and Jurkat). * = $p < 0.05$.

Overexpression of microRNAs

To investigate whether overexpression of single microRNAs results in reduced surface expression of ULBP1, we transiently transfected HeLa and Jurkat cells with 200 ng of pSuper vectors, containing an expression cassette to generate artificial microRNA precursors. In HeLa cells (Fig. 30A), expression of miR-140-5p, miR-409-3p and miR-650 was 98 to 726 fold higher in cells transfected with overexpression plasmid compared to empty pSuper control vector. For miR-380-3p, miR-381 and miR-578, the miR levels were at least 2067 to 10027 fold increased. Overexpression of miR-140-5p and miR-650 in Jurkat cells (Fig. 30A) resulted in a 36 and 282 fold increase of miR expression levels. For miR-380-3p, miR-381,

miR-409-3p, miR-433-3p and miR-578, expression was at least 25 to 1083 fold increased. Despite high expression of microRNAs in Jurkat cells, no effect on ULBP1 cell surface expression was observed (Fig. 30B).

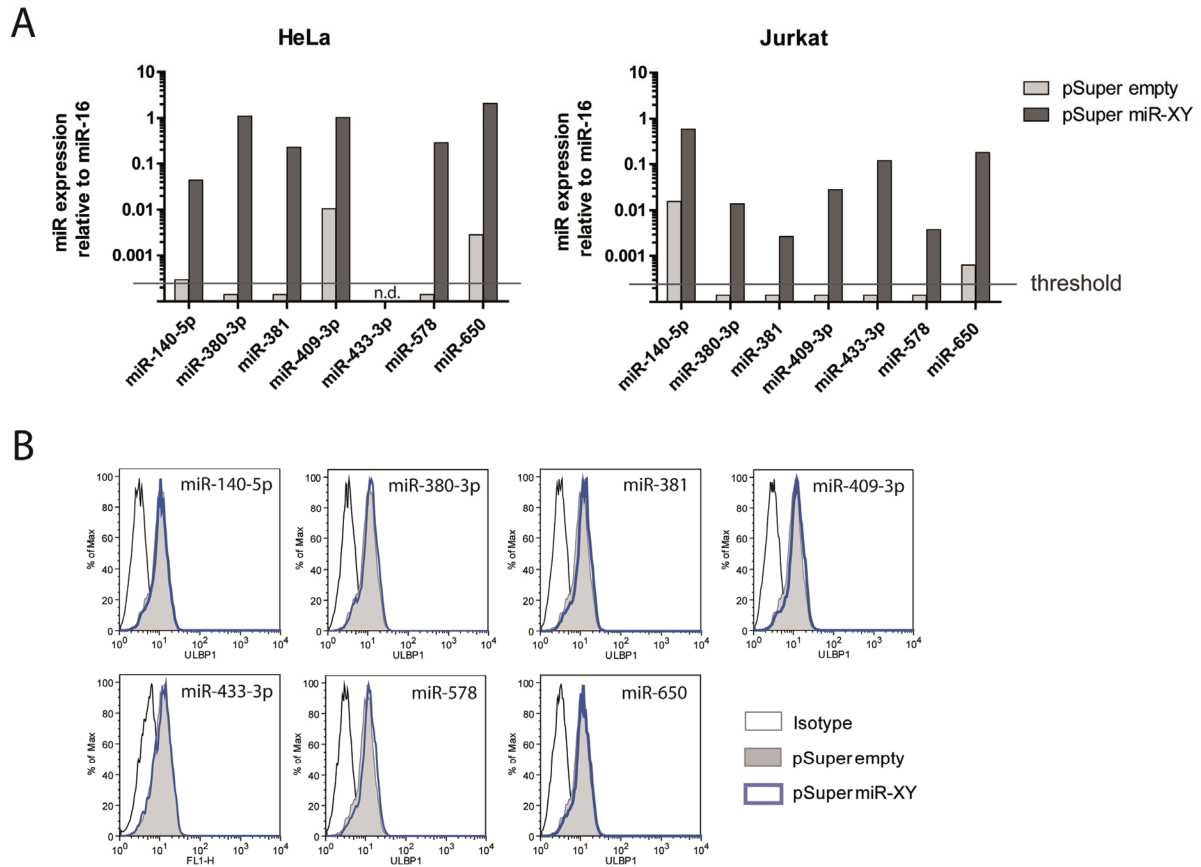

Figure 30: Overexpression of candidate microRNAs. (A) expression levels of candidate microRNAs upon transient transfection of microRNA overexpression vectors (dark grey) or control vectors (light grey) into HeLa and Jurkat cells. (n=1), n.d.= not done. (B) Effect of microRNA overexpression on the surface protein level of ULBP1 in Jurkat cells (n=1).

Since mRNAs can be targeted by multiple microRNAs[178], we investigated whether overexpression of the seven candidate microRNAs affects the surface expression of ULBP1. 50 ng of each overexpression vector were simultaneously transfected into Jurkat cells. The increase of microRNA expression level (Fig. 31A) upon transfection ranged from 2.5-fold (miR-381) to 252-fold (miR-650), and no upregulation was obtained for miR-578. Overexpression of multiple microRNAs did not decrease the ULBP1 surface expression (Fig. 31B). Consistent with this observation, co-transfection of all miR-overexpression vectors with

luciferase reporters did not change luciferase activity compared to co-transfection with pSuper empty (Fig. 31C). Based on this results, we were unable to demonstrate a specific modulatory role of the seven candidate microRNAs in the regulation of the regions U1-6 and U1-9 of the ULBP1-3'UTR.

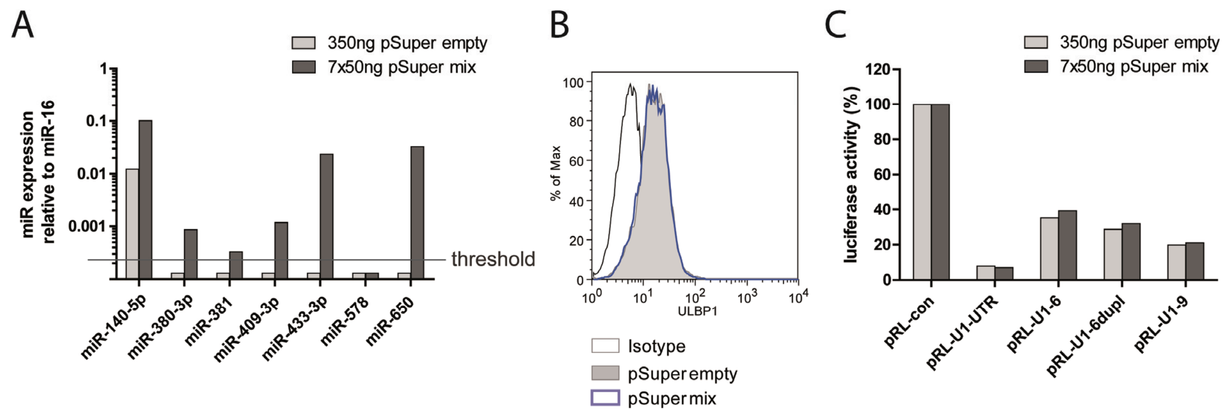

Figure 31: Simultaneous overexpression of seven candidate microRNAs. (A) MicroRNA expression levels were measured upon transient transfection of 350ng pSuper empty (light grey) or co-transfection of seven microRNA overexpression vectors (7x50ng pSuper mix; dark grey) into HeLa cells. Results are means ± SEM of duplicates. (B) FACS analysis of ULBP1 surface expression in Jurkat cells transfected with pSuper empty (grey shaded histogram) or pSuper mix (thick black line). Thin grey line: staining with isotype control Ab and secondary antibodies. (C) Luciferase activity was measured upon co-transfection of 350ng pSuper empty vector (light grey) or 7x50ng pSuper mix (dark grey) with luciferase reporter vectors. Luciferase activity was normalized to the activity of pRL-con. Results are means ± SEM of duplicates.

Downmodulation of miR-140-5p by antagomirs

The function of endogenous microRNAs can be transiently antagonized by chemically modified oligonucleotides, complimentary to individual microRNAs.[255] To answer the question, if neutralizing of miR-140-5p, which is highly expressed in HeLa and Jurkat cells, diminished the high reduction in luciferase activity, we co-transfected luciferase vectors together with antimir-140-5p into HeLa and Jurkat cells (Fig. 32). No increase of luciferase activity was observed in cells transfected with antimiR-140-5p. To show, that antagomirs are transfectable into HeLa and Jurkat cells, control let-7a-antagomirs were co-transfected with a let-7 luciferase construct[245] into HeLa and Jurkat cells, resulting in efficient downregulation of HeLa and Jurkat (data not shown). Therefore we conclude, that miR-140-5p is most likely not involved in regulation of ULBP1 expression.

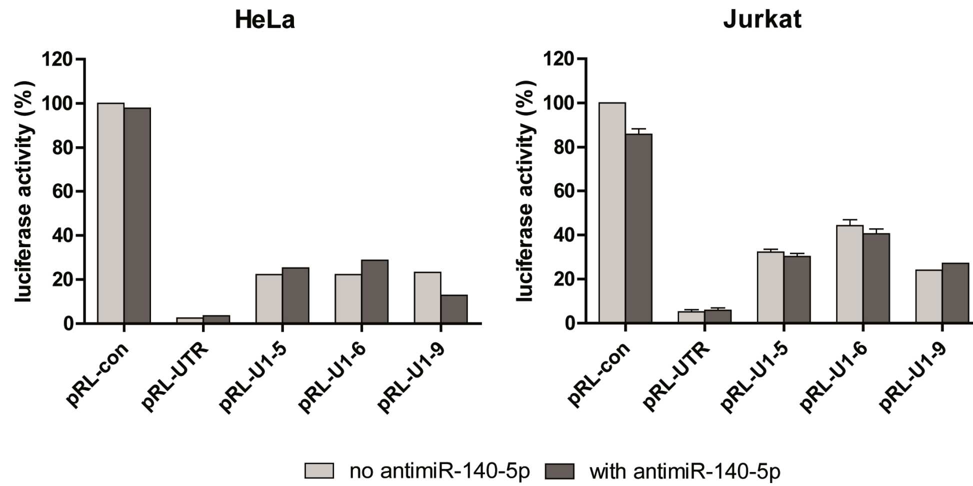

Figure 32: Silencing of endogenous miR-140-5p by antimiR-140-5p. Luciferase activity was measured in HeLa and Jurkat cells upon co-transfection of reporter constructs with 100 nM of antimiR-140-5p (dark grey). As control, tranfection of reporter construct was performed without antimiR (light grey). Results are means ± SEM of duplicates. (n=1-3).

Knockdown of Drosha

Since investigation of specific candidate microRNAs by functional assays did not identify specific microRNAs, which are potentially involved in regulation of ULBP1, we used an approach to knock-down Drosha, a crucial component of the microRNA biogenesis pathway.[256] HeLa cells were transduced with a lentiviral construct containing a shRNA against Drosha (LV-shDrosha) or a control shRNA (LV-shControl), and the knockdown efficiency of Drosha as well as the surface expression and mRNA levels of ULBP1 and MICA/B was assessed seven days post transduction. According to the number of GFP^+ cells, transduction efficiency was 93% and 86% for cells transduced with LV-shDrosha and LV-shControl, respectively (data not shown). In cells transduced with LV-shDrosha, the Drosha mRNA level was 2.7 fold reduced compared to LV-shControl. Disruption of the microRNA machinery increased the level of MICA transcripts by 1.4 fold (Fig. 33A) and lead to an upregulation of MICA/B surface protein expression (MFIR(LV-shControl) = 11.7 vs. MFIR(LV-shDrosha) = 17.1; Fig. 33B), which is in accordance with previous reports.[95] However for ULBP1, no increase in surface expression was observed and the amount of ULBP1 mRNA even decreased 1.7 fold upon knock-down of Drosha (Fig. 33. A and B). Accordingly, when cells were transduced with LV-RL-U1-UTR, Drosha knock-down had no effect on luciferase activity, which remained at a similarly low level of approximately 10% as in cells transduced with LV-shControl (Fig. 33C).

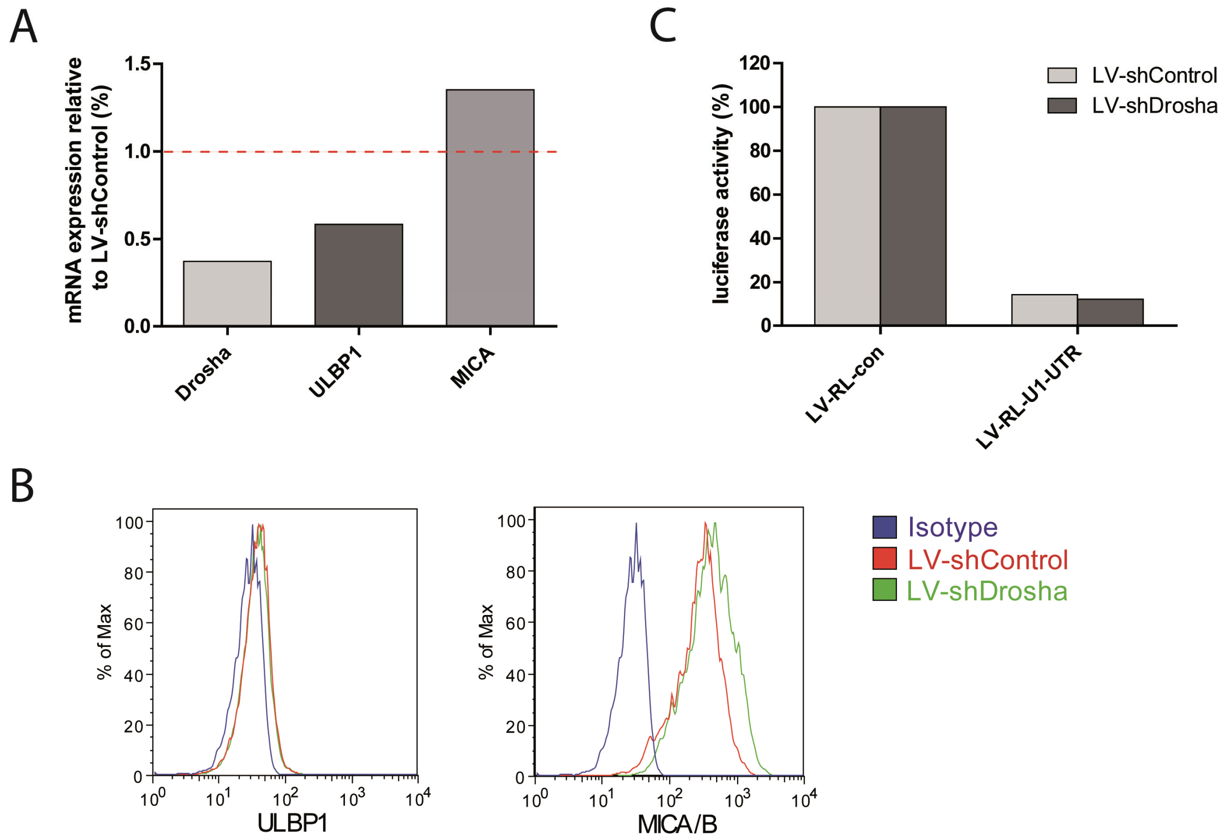

Figure 33: Downregulation of Drosha with short hairpin RNA in HeLa cells. (A) Measurement of Drosha, ULBP1 and MICA mRNA levels upon transduction with LV-shDrosha. The expression level of each mRNA was normalized to the corresponding mRNA expression levels upon transduction with a control LV-shCon. Results are means of duplicates (B) FACS analysis of ULBP1 and MICA/B surface expression on HeLa cell upon transduction with LV-shControl (red) or LV-shDrosha (green). Staining with isotype control Ab and secondary antibodies is indicated in blue (C) The luciferase activity was measured upon co-transduction of LV-shControl (light grey) or LV-shDrosha (dark grey) and lentiviral luciferase reporter constructs (LV-RL-con and LV-RL-U1-UTR). Transduction efficiency was monitored by measurement of GFP^+ cells by FACS. Luciferase activity was normalized to the amount of GFP^+ cells and then normalizes to the luciferase activity of the control vector (LV-RL-con). Results are means of duplicates.

As an alternative approach, we performed knock-down of Drosha by using a lentiviral vector containing a DOX-inducible shDrosha cassette.[252] The transduction efficiency in HeLa cells was 88%, as indicated by the percentage of GFP^+ cells (Fig. 34A). Eight days post induction of shDrosha by DOX treatment, the transcript level of Drosha was 2.9 fold reduced. In accordance with the previous result, knock-down of Drosha lead to a 3.3 and 2.9 fold increase of MICA and MICB mRNA, respectively, and induced MICA/B surface expression on GFP^{high} cell 3-fold (Fig. 34 B and C). However, ULBP1 surface protein level was unaffected

and ULBP1 transcript was 2.0 fold reduced (Fig. 34 B and C). The fact that the knock-down of Drosha was sufficient to increase MIC molecules but not ULBP1 expression may indicate that ULBP1 is regulated by mechanisms other than microRNAs.

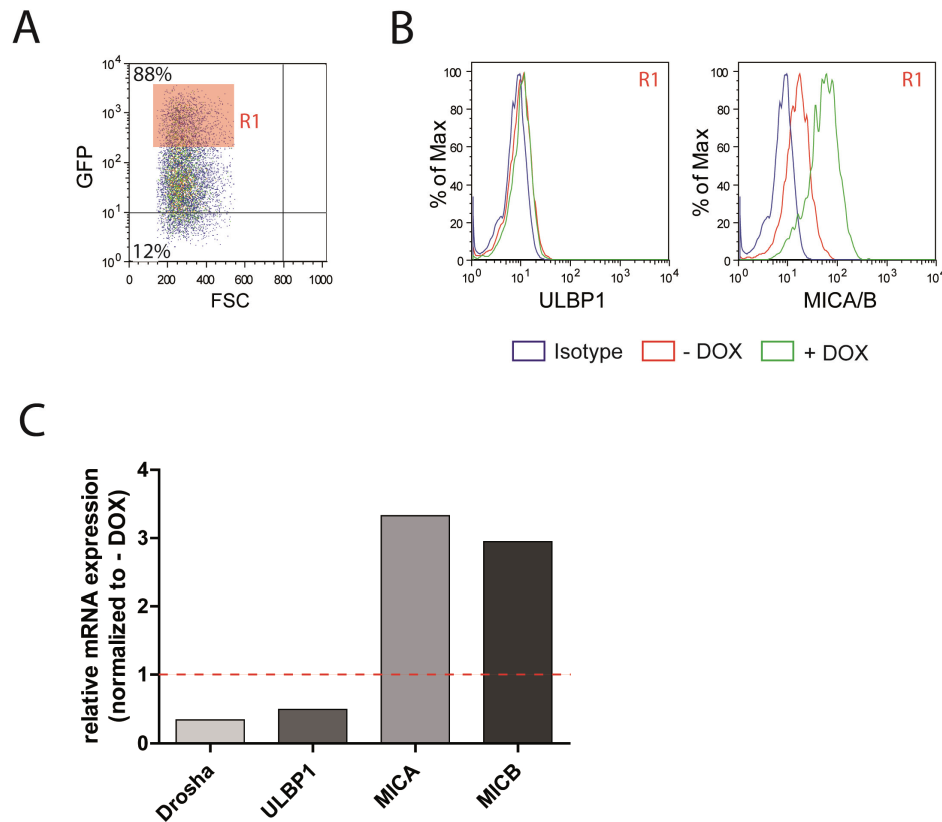

Figure 34: DOX-induced downregulation of Drosha with short hairpin RNA in HeLa cells. (A) FACS analysis of transduction efficiency by measurement of GFP^{+} cells upon transduction with DOX-inducible LV-shDrosha (B) Measurement of ULBP1 and MICA/B surface expression on HeLa cell upon transduction with DOX-inducible LV-shDrosha. Transduced cells were culture for 8 days with (green) or without DOX (red). Staining with isotype control Ab and secondary antibodies is indicated in blue. (C) Measurement of Drosha, ULBP1, MICA and MICB mRNA levels upon transduction with DOX-inducible LV-shDrosha. Transduced cells were culture for 8 days with DOX. mRNA levels were normalized to corresponding cells without DOX treatment.

Overexpression of ULBP1 in HeLa and Jurkat

Since knockdown of Drosha did not result in upregulation of ULBP1 surface expression, we wanted to exclude that intrinsic mechanisms in HeLa cells prevent the presentation of ULBP1 protein on the cell surface. Therefore, HeLa and Jurkat cells were transiently transfected with the ULBP1 overexpression vector RSV.5ULBP1 or the control vector RSV.5neo. Upon transfection of HeLa cells with 200ng and 400 ng RSV5.ULBP1, respectively, 24% and 41% of cells were expressing high levels of ULBP1 (Fig 35). Transfection of Jurkat cells with RSV5.ULBP1 resulted in 5% and 9% of ULBP1 positive cells, respectively. No expression was observed in cells transfected with RSV.5neo (data not shown). Therefore we conclude that HeLa and Jurkat cells are capable to express high levels of ULBP1 surface protein.

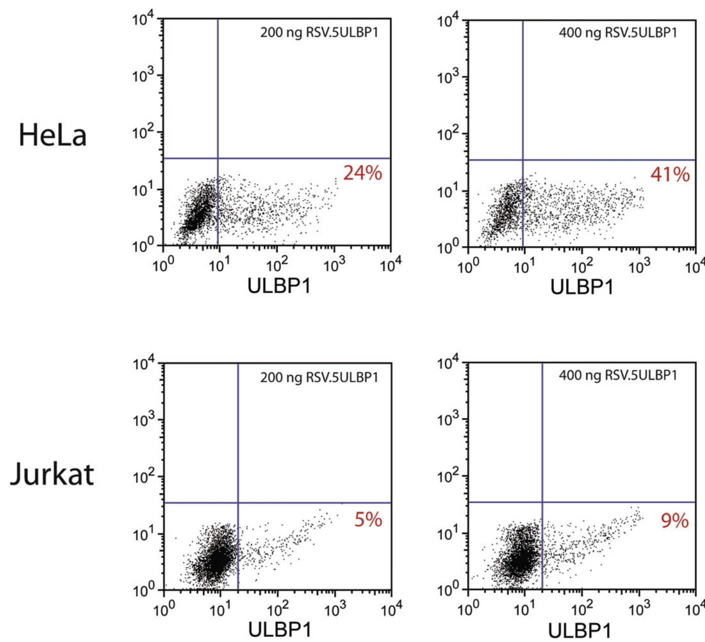

Figure 35: Overexpression of ULBP1 in HeLa and Jurkat cells. Measurement of ULBP1 cell surface expression upon transient transfection with 200ng or 400ng of RSV.5ULBP1

1.4 Discussion

The activating NKG2D ligand ULBP1, plays an important role in recognition of tumor-transformed target cells by cytotoxic NK cells.[37] The expression of ULBP1 must be tightly regulated to ensure, that tumor cells are attacked and eliminated whereas healthy cells are spared.[56] The mechanisms responsible for the regulation of ULBP1 expressions are poorly understood. Based on studies with other molecules of the NKG2D-L family, these ligands can be regulated not only by transcriptional, but also by post-transcriptional and post-translational mechanisms.[62] These mechanisms have been well documented in case of MIC ligands, were both transcriptional upregulation as well as post-transcriptional mechanisms involving 3'UTR and also protein shedding from the cell surface have been described.[257] Previous studies on ULBP1 showed the transcriptional upregulation involving regulation at the putative promoter region.[258] Our own studies have shown, that post-translational regulation by shedding is not strongly pronounced in case of ULBP1.[156] Whether this ligand is a subject of post-transcriptional regulation has not yet been investigated. Since ULBP1 carries a 2.4kb-long 3'UTR, containing 4 ARE motifs and more then 200 microRNA binding sites, our aim was to investigate the involvement of post-transcriptional mechanisms in regulation of ULBP1 expression.

1.4.1 Involvement of 3'UTR in regulation of ULBP1

In order to investigate the role of ARE motifs and microRNA binding sites in regulation of ULBP1 expression, we established a transient and a stable lentiviral reporter system based on luciferase reporter constructs with and without the full-length ULBP1-3'UTR. Transfection or transduction of the 3'UTR containing reporter constructs into four different human cancer cell lines (HeLa, Jurkat, K562, Raji) and into primary human fibroblasts (HFF) resulted in a highly pronounced and reproducible reduction of luciferase activity. Compared to cells transfected with the control construct pRL-con, the luciferase activity was reduced to 7 – 38% in cells transfected with the reporter construct containing the full-length ULBP1-3'UTR (Fig. 20). With these experiments we provided the first evidence, that the 3'UTR of ULBP1 is functionally relevant and may therefore be involved in regulation of ULBP1 expression.

Fragmentation of regulatory elements and investigation by luciferase assays is a well accepted method, which is often applied in promoter studies and was also used to investigate the 3'UTR of candidate genes.[200,258] In order to locate regions with high ULBP1 regulatory capability, we generated nine reporter vectors containing fragments of the ULBP1-3'UTR of different sizes. Upon transfection of these contructs into HeLa and Jurkat cells, a strong reduction in luciferase activity was observed for all 3'UTR fragments, ranging from 19 – 61% (Fig. 23). This finding suggests, that the elements which are responsible for regulation of ULBP1, are distributed over the entire 3'UTR. Interestingly, the luciferase activity of the single fragments was never as low as observed with the full-length constructs suggesting, that multiple regulatory elements in different parts of the 3'UTR contribute to the regulation of ULBP1.

The reporter constructs which are being used in luciferase assays often cover a wide range of sizes. Investigations of the influence of the size of luciferase vectors on the luciferase activity upon transient transfection showed, that the luciferase activity inversely correlates with the construct size.[259] In our study the difference in size between the control vector pRL-con (6.3kb) and the full size vector pRL-U1-UTR (8.7kb) is 2.4 kb and one could argue that the strongly reduced luciferase activity upon transient transfection of the construct containing the full-length 3'UTR might be caused by this difference in size. However, the diminished luciferase activity was not only observed in the transient reporter system, but a similar reduction was also observed in the lentiviral reporter system. Furthermore, a low luciferase activity was also found for smaller constructs carrying fragments of the 3'UTR. Therefore, reduction in the luciferase activity upon transient transfections cannot be solely attributed to a diminished transfection rate of pRL-U1-UTR due to its enlarged size compared to the pRL-con vector, although we cannot rule out that the size of the constructs may have influenced the luciferase activity to a certain degree also in our experiments.

The reduction in luciferase activity which we observed upon transfection or transduction of constructs containing the full-length ULBP1-3'UTR or its fragments might be a consequence of mRNA degradation or translational repression. Degradation of mRNA can be caused by ARE- and microRNA-mediated regulation. Translational repression by storage of mRNAs in special cellular compartments, the P-bodies, has been linked to microRNA-mediated regulation.[189] In order to investigate which mechanism may regulate ULBP1 expression, we measured the mRNA levels of reporter constructs containing the full-length ULBP1-3'UTR or the 3'UTR fragments by qPCR and correlated the mRNA levels with the corresponding luciferase activity in transfected cells. In Jurkat cells, the mRNA levels of

reporter transcripts ranged from 31 – 110% compared to the mRNA level upon transfection with the control vector, whereas the corresponding luciferase activity ranged from 7 – 40% (Fig. 24). The observation that the mRNA levels were decreased more moderately then the luciferase activity might indicate that translational repression causes the reduction in luciferase activity in Jurkat cells. In contrast, in HeLa cells, the mRNA levels of reporter transcripts were decreased dramatically, ranging from 6 – 22% (Fig. 24), which suggests that the reduced luciferase activity is a consequence of mRNA degradation. These results imply, that different regulatory mechanisms might regulate the expression of ULBP1 in HeLa and in Jurkat cells.

Our findings that multiple regulatory elements in various parts of the 3'UTR may contribute to regulation of ULBP1 expression and that the regulatory mechanism my differ depending on the cell- or tissue-type, suggest a complexity in the process of post-transcriptional control of ULBP 1 expression. Below, we discuss our data on the involvement of ARE motifs and microRNA-dependent regulation at ULBP1-3'UTR.

1.4.2 Role of ARE in regulation of ULBP1 gene expression.

Analysis of the ULBP1-3'UTR has revealed, that the 3'UTR harbours four ARE motifs in two regions, U1-5 and U1-9. In order to investigate the role of ARE sites in regulation of ULBP1 gene expression, we mutated the AUUUA motifs by substitution of one or three bases, and performed luciferase reporter assays. Upon transfection of constructs harbouring the mutated fragments, the low luciferase activity seen with unmutated vectors, was not increased (Fig 21). This indicates that the pronounced reduction of luciferase activity upon transfection of pRL-U1-UTR or the unmutated pRL-U1-5 and pRL-U1-9 constructs (Fig. 23) is not caused by ARE-mediated degradation of reporter transcripts. Since mutating of ARE motifs did not enhance luciferase activity in both HeLa and Jurkat cells, the differential effect of ARE in these two cell types in unlikely. Therefore we conclude, that ARE elements are not involved in negative regulation of ULBP1 expression.

Interestingly, the luciferase activity was even further reduced upon transfection with mutated reporter constructs, ranging from 61-88% compared to unmutated fragments. This observation suggests, that ARE sites in the ULBP1-3'UTR might serve as stabilizing elements. Indeed, ARE sites do not only have mRNA destabilizing properties. The function of

ARE elements is highly dependent on the sequence composition around the ARE motifs and on the type of *trans*-acting ARE-binding proteins which attach to the ARE site.[242] Binding of the ARE-binding protein HuR and other proteins to ARE results in stabilization of some transcripts.[170,242] The question, whether also ULBP1 mRNA is stabilized by these proteins could be anwered using specific inhibitors[260] or siRNA[261] against HuR or other proteins. Changes in half-life of transcripts upon inhibitions of ARE-binding proteins could be examined by treatment of cells with the transcriptional inhibitors Actinomycin D (ActD) or 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) and subsequent measurement of mRNA levels by qPCR at several time points.[242,261]

Another explanation for the reduced luciferase activity upon mutation of ARE sites might be, that the mutation of U1-5 and U1-9 by base pair substitution leads to unintentional generation of new microRNA binding sites. Computational analysis of the unmutated and mutated U1-5 fragments revealed, that the substitution of one nucleotide (ATTTA to ATGTA) in the three ARE motifs of U1-5 indeed generated a second microRNA binding site for miR-496 (analysis was kindly done by N. Shomron, Tel Aviv, Israel). However, using microRNA qPCR, we were unable to detect miR-496 expression in Hela and Jurkat cells and hence it is unlikely, that the additional binding site for miR-496 contributed to the reduction of luciferase activity in cells transfected with the mutated construct pRL-U1-5-AREmut1. However, we cannot exclude that certain *de novo* microRNA binding sites for other microRNAs were generated by mutation of U1-5 and U1-9 and may have an impact on the luciferase activity.

1.4.3 Role of microRNAs in regulation of ULBP1 expression

Computational algorithms have predicted that more than 200 putative microRNA binding sites are located in the 3'UTR of ULBP1. In order to investigate the functional role of microRNAs in regulation of ULBP1, we selected 13 candidate microRNAs which were predicted to target the region U1-6 and/or U1-9 at least once. Studies in which the role of microRNAs in regulation of particular genes has been documented rely on approaches, which involve the analysis of the seed sequence[184], overexpression or silencing of microRNAs[227,262], as well as analysis of the involvement of enzymes involved in miR biogenesis, such as Drosha.[95] These approaches were applied in our work.

In order to examine the expression level of the 13 candidate microRNA, we performed the microRNA qPCR and normalized the microRNA expression levels to the housekeeping miR-16. This analysis revealed, that 6 out of 13 candidate microRNAs were indeed expressed in HFF, 4 microRNAs were expressed in HeLa and only 2 microRNAs were expressed in Jurkat cells (Fig. 28). The low number of expressed microRNAs in the cancer cell lines HeLa and Jurkat may reflect the observation, that the microRNA expression is globally decreased in many tumors.[193] Among the three types of cells used here, the 6 microRNAs (miR-140-5p, miR-381, miR-409-3p, miR-433-3p, miR-496, miR-650) were expressed in an overlapping manner. These data indicate, that only 6 of the 13 candidate microRNAs which are expressed in HeLa, Jurkat or HFF can be responsible for the pronounced reduction of luciferase activity upon transfection with the full-length ULBP1-3'UTR reporter. It remains open, whether the remaining 7 candidate microRNAs which were not expressed in HFF, HeLa and Jurkat, might be involved in regulation of ULBP1 in other cell types.

In order to investigate, if miR-140-5p, miR-409-3p, miR433-3p and miR-650 are involved in regulation of ULBP1 expression, we simultaniously mutated the seed sequences of these candidate microRNAs in fragment U1-6 by base pair substitutions or deletions. Upon transfection of luciferase constructs containing the mutated seed sequences into HeLa and Jurkat cells, the luciferase activity was increased to 110–119% compared to unmutated construct (Fig. 29). Even though the effect was small, it was reproducibly seen in every experiment suggesting, that these endogenously expressed microRNAs might at least partially contribute to the regulation of ULBP1 expression.

Based on these data, we continued the studies by an alternative approach of overexpression of selected candidate microRNAs and investigated, whether such overexpression can reduce the surface expression of ULBP1. Even though the microRNA expression levels were up to 10000 fold increased compared to the endogenous microRNA levels, this overexpression did not decrease the ULBP1 surface expression in ULBP1$^+$ Jurkat cells. There was no effect on the expression of ULBP1 and no decrease in luciferase activity, irrespective whether overexpression was carried out for single candidate microRNAs or for their combinations. These data imply, that the candidate microRNAs we have overexpressed are not involved in regulation of ULBP1 expression.

However, we cannot exclude a possibility that overexpression of microRNAs was not strong enough. In our hands, simultaneous transfection of multiple microRNA overexpression vectors resulted in a more moderate upregulation of microRNAs (2 – 250 fold), compared to transfection of single overexpression vectors (24 – 10000 fold) (Fig. 30 and 31). Mandelboim

and colleagues have reported, that overexpression of microRNAs in their hands had only an effect on the surface expression of the target protein when the transduction efficiency of their lentiviral constructs and in consequence the expression level of candidate microRNAs was high.[95] Furthermore, we can not exclude, that the lack of an effect on ULBP1 surface expression upon overexpression of candidate microRNAs might be due to an alteration of the ULBP1-3'UTR in Jurkat cells. A recent study has shown that proliferating cells express transcripts with shortened 3'UTRs due to alternative cleavage and polyadenylation, and that these alterations result in fewer microRNA target sites.[263] Interestingly, a sequence analysis of the ULBP1-3'UTR by W. Lutz (German Primate Center, Primate Genetics Laboratory, Göttingen, Germany) revealed, that the 3'UTR contains multiple canonical and alternative poly(A) sites (personal communication). Investigation of the ULBP1-3'UTR by Northern Blot, 3'RACE-PCR or sequencing could answer the question, if the 3'UTR is shortened in Jurkat, as well as in other cell types or tissues.

Another approach to investigate involvement of microRNAs in gene regulation is the silencing of endogenous microRNAs by antisense microRNAs. In order to investigate if miR-140-5p, which is highly expressed in both HeLa and Jurkat cells, contributes to the regulation of ULBP1, we co-transfected the reporter constructs and antimiR-140-5p into HeLa and Jurkat cells. Measurement of luciferase activity revealed, that silencing of endogenous mir-140-5p had none or only a moderate effect on luciferase activity (Fig 32). Therefore we suggest, that miR-140-5p is not involved in regulation of ULBP1 expression. Silencing of other microRNA including miR-381, miR409-3p, miR-433-3p, miR-496 and miR-650, all which are expressed in HeLa, Jurkat and/or HFF, might allow to exclude the involvement of these microRNAs in regulation of ULBP1 expression, but we did not perform these analyses due to technical restrictions.

In summary, we can conclude that the functional assays we have performed did not answer the question whether the seven investigated candidate microRNAs (miR-140-5p, miR-380-3p, miR-381, miR-409-3p, miR-433-3p, miR-578 and miR-650) are involved in regulation of ULBP1. However, further investigations of these candidate microRNAs by experiments suggested above, could help to clarify the role of these candidate microRNAs in regulation of ULBP1.

1.4.4 Role of the microRNA biogenesis pathway involving Drosha

Although we were not able to identify the involvement of specific microRNAs, we have to emphasize that none of the results we have obtained by luciferase assays and functional assays disproved the possibility that microRNAs are involved in regulation of ULBP1. In order to collect further arguments to support this hypothesis we investigated, whether disruption of the microRNA biogenesis pathway would affect the mRNA levels and the surface expression of ULBP1. Downregulation of Drosha by shRNA, using two different lentiviral systems, resulted in increased mRNA levels and surface expression of MIC molecules, which served as a positive control. However no upregulation of mRNA or surface protein was observed for ULBP1 (Fig. 33 + 34). Instead, the mRNA level of ULBP1 mRNA was even decreased upon knockdown of Drosha. Furthermore, the luciferase activity did not increase upon co-expression of Drosha-shRNA and luciferase reporter constructs. Therefore also this approach did not help to proof, that microRNAs are involved in regulation of ULBP1.

Multiple studies have shown that knock-down of Drosha results in a decline of pre-microRNAs and mature microRNAs.[264-265] Although we have not examined the microRNA levels in HeLa cells upon knock-down of Drosha we assume, that the level of microRNAs was reduced. This assumption is supported by the facts, that the Drosha mRNA level was ~3 fold diminished in cells transduced with shDrosha constructs. Furthermore, the expression of MIC transcript, a validated microRNA target[95], was increased. Therefore we speculate, that the decrease of cellular microRNAs was sufficient enough to affect regulation of MIC molecules but might not be sufficient enough to upregulate ULBP1 expression. The observation that the effect of Drosha knock-down on ULBP1 and MIC transcripts was divergent might indicate, that these NKG2D-L are regulated by different mechanisms. One possible explanations for the decline of ULBP1 mRNA level upon knockdown of Drosha could be, that the putative low microRNA level in Drosha-deficient HeLa cells might have caused increased expression of microRNA-regulated RNA-destabilizing proteins, and in consequence degradation of ULBP1 mRNA.

Taken together these data indicate that further experiments are needed to clarify if microRNAs are involved in regulation of ULBP1 expression. An alternative approach to the knock-down of Drosha might be knock-down of Dicer by shRNA, another crucial enzyme in the microRNA biogenesis pathway. In HEK293 cells, knock-down of Dicer leads to an increase of mRNA transcripts, containing 3'UTRs with putative microRNA binding sites.[266]

Knock-down of Dicer might lead to a more efficient reduction of microRNA levels and therefore might increase ULBP1 mRNA levels.

Another possibility to investigate whether ULBP1 is regulated by microRNA might be co-immunoprecipitaion of Argonaute (Ago) proteins and Ago-associated mRNAs. Ago is part of the miRISC complex and guides microRNAs to the 3'UTR of the target mRNA. By using this approach, G. Meister and colleagues were able to isolate Ago-associated mRNAs and experimentally validated six mRNAs as microRNA targets[267]. In order to investigate whether ULBP1-3'UTR-containing luciferase reporter transcript are associated with Ago2, we co-transfected in a preliminary experiment an Ago2-overexpressing plasmid and ULBP1-3'UTR luciferase reporter constructs into Jurkat cells and performed co-immunoprecipitation of Ago2, followed by RNA isolation of co-precipitated transcripts. Ago2-associated luciferase reporter mRNA was measured by qPCR, using luciferase-specific primers. However, the results gained from this experiment were not conclusive. A refinement of experimental set-up might lead to definite results in order to clarify, if microRNAs are involved in regulation of ULBP1 expression.

1.4.5 Computational prediction and selection of candidate microRNAs

Our attempts to identify functional relevant microRNAs in regulation of ULBP1 expression have failed. This however does not exclude, that microRNAs are involved in regulation of ULBP1. Beside the already discussed drawbacks in our functional assays, a major drawback for our failure might be the computational analysis and the strategy which was used to select for candidate microRNAs, involved in regulation of ULBP1 expression. Computational prediction by using several algorithms has revealed, that the ULBP1-3'UTR contains more then 200 microRNA binding sites. The selection of 13 candidate microRNA, all which were predicted to target region U1-6 and/or U1-9 in the ULBP1-3'UTR, were fully based on predictions resulting from Targetscan and other algorithms.

Although computer algorithms are widely used to predict candidate microRNAs and are a well accepted method to unravel the role of microRNAs in biological processes and diseases, the prediction quality of current algorithms is poor.[184,268] The major challenge in developing algorithms is the fact, that most of mammalian microRNAs recognize their mRNA targets via partial complementarity and therefore simple homology-based searches can not be

applied to uncover microRNA targets.[188] Therefore the algorithms are mainly based on informations, gained through systematic target-site mutation experiments and extensive bioinformatical analysis.[269-271] Based on these approaches, the main prediction parameters used in most of the current algorithms are stringent or moderately stringent pairing of the microRNA ‚seed' sequence to a complementary site in the target mRNA, and evolutionary conservation of potential microRNA binding sites in the 3'UTRs among different species. Additional parameters for prediction include good structural accessibility, nucleotide composition, and thermodynamic stability as well as location, number and proximity of the microRNA binding sites along a target mRNA.[178,268]

To assess the quality of current prediction algorithms, Alexiou and colleagues compared the results from 9 prediction algorithms against experimental results gained from a high-throughput approach.[268] Investigation of 5 microRNAs revealed, that five of nine programs (Diana-microT 3.0, Targetscan 5.0, TargetscanS, Picta, and ElMMo) had a precision of ~50% (50% of the computational results were correct when compared with experimental results; 50% false positive results). The sensitivity of these programs ranged from 6-12% (only ~10% of experimentally validated targets were correctly predicted by computational analysis; 90% false negative results). All these programs heavily rely on the evolutionary conservation of the seed region. The precision of the remaining four algorithms was even weaker. The authors conclude that even the most sensitive programs fail to identify large parts of target genes. Since the ULBP1-3'UTR is not conserved among species we can assume, that the prediction results we have gained by using different algorithms are not of a good quality, due to a high rate of false negative (experimentally supported microRNA-target gene interaction that are not predicted by the algorithm) and false positive (mistakenly predicted; microRNA:target-interaction experimentally disproven) predictions.

Recently developed technologies will help to revise and improve the currently existing algorithms. Beside direct validation of microRNA:target-interactions such as luciferase reporter assays or seed sequence mutations, high throughput methods for indirect validation play an important role in this process. Overexpression or silencing of microRNAs and subsequent monitoring through gene expression arrays can help to identify microRNA targets, where binding of microRNAs results in changes of mRNA level. Recently developed high-throughput proteomic techniques allow to identify also microRNA targets, which are translationally repressed by microRNAs and where the mRNA level is not affected.[272-274] Two other recently developed methods to identify microRNA targets are the already mentioned immunoprecipitation of RISC components[267,275] and crosslinking immunoprecipitation

(HITS-CLIP), followed by high-throughput sequencing of RNAs.[276] However a common problem of all these methods are secondary and nonspecific effects.[268]

Not only the quality of prediction algorithms might be responsible for the fact that we failed to identify functional relevant microRNAs, but also the strategy we have chosen to select 13 candidate microRNAs. Computational prediction revealed that more then 200 microRNA might target the ULBP1-3'UTR. Examination of all predicted candidate microRNAs would have required high-thoughput approaches like microRNA microarrays. Instead, our strategy was to narrow down the number of predicted microRNA binding sites by generation of luciferase constructs containing fragments of the 3'UTR in order to uncover regions in the ULBP1-3'UTR with high regulatory capacity. Fragmentation of regulatory elements is a commonly used approach to investigate promoters and was also reported for investigation of the 3'UTR of candidate genes.[200,258] Based on the results of luciferase reporter assays, region U1-6 and U1-9 were considered as regions with high regulatory potential. Therefore, we concentrated our studies only on U1-6 and U1-9 although we know, that regulatory elements are most likely located also in other parts of the ULBP1-3'UTR. This fact implies, that we have disregarded many microRNAs which might play a role in regulation of ULBP1.

Furthermore, we did not examine all predicted candidate microRNAs in region U1-6 and U1-9, but perfomed functional assays only with a selection of candidate microRNAs. The selection process was performed at two different time points. The coincidence, that the Targescan algorithm was revised during the course of our study forced us to use two different versions of the Targetscan (V4.2 and V5.2). When comparing the output of V4.2 and V5.1, the results differed significantly (see also Table 11). Whereas 7 microRNAs were predicted with V4.2 to target U1-6, the number of microRNAs in the revised algorithm V5.1 increased to 11. However, prediction results of only 4 microRNAs (miR-380-3p, miR-548c, miR-612, miR-657) were overlapping. Furthermore, one microRNA was predicted only with V4.2 and seven microRNAs only with V5.1. One microRNA was predicted with V4.2 to target the U1-6, while V5.1 assigned this microRNA to target U1-9. One microRNA predicted with V4.2 was afterwards removed from the microRNA registry, since this alleged microRNA was identified as a fragment of U11 splisosomal RNA. These differences in prediction by using two versions of Targetscan illustrate, that the development of prediction algorithms is an active and ongoing field of research and that the results, obtained from such algorithms can only be used with caution.

In the second round of selection, we combined and compared the results, which we obtained by using Targetscan V4.2 and V5.1, Elmmo3, microInspector V1.5 and DIANA microT V3.0 in order to increase the chance to select physiologically relevant candidate microRNAs. Based on this strategy, 7 additional candidates were selected. However a recently published study revealed, that one accurate algorithm might be better then the combination of predictions performed with several algorithms.[268] Therefore it is questionable, if we would really increase the accuracy of our prediction by combining multiple algorithms.

In our study, the expression of candidate microRNAs by qPCR was measured only after the selection process has been performed, as based on the one hand on computational analysis and on the other hand on the choice of putative 3'UTR fragments with regulatory function. Of thirteen candidate microRNAs, only 2 and 4 microRNAs turned out to be expressed in Jurkat and HeLa cells, respectively. Since only microRNAs, which are endogenously expressed in HeLa and Jurkat can cause the prominent reduction of luciferase activity, which was observed upon transfection with all luciferase reporter constructs containing parts of the ULBP1-3'UTR, we have finally analysed only 2 and 4 microRNAs, which might be relevant in regulation of ULBP1 expression in Jurkat and HeLa cells, respectively. The fact that the other candidate microRNAs are not expressed in HeLa and Jurkat does not exclude the possibility, that these microRNAs regulate ULBP1 expression in other cell types. Expression analysis in HFF has revealed, that these cells express 6 out of 13 candidate microRNAs. Furthermore, microRNA array experiments have shown, that the microRNA signature differs considerably among different cell lines and cell types.[193,225]

In a retrospective view, we might have increased the chance to discover functional relevant microRNAs by selection of candidate microRNAs based on microRNA microarray approaches. In a first step, examination of the microRNA expression pattern in HeLa, Jurkat and HFF would allow to exclude in advance these microRNAs from functional studies, which are not expressed in all three cell lines. In a second step, comparison of microRNA expression patterns would allow to select candidate microRNAs, which are expressed in all three cell lines. However one has to keep in mind, that not nessecarily the same microRNAs might be involved in regulation of ULBP1 in the different cell lines, and candidate microRNAs might be disregarded by focusing only on microRNA which are commonly expressed by all three cell lines. In a third step, the selection of candidate microRNAs could be further restricted by comparing the results obtained from microarrays and computational prediction algorithms.

1.4.6 Conclusions

In this thesis, we provide the first evidence that the 3'UTR of ULBP1 is involved in regulation of ULBP1 gene expression. Furthermore we were able to exclude that the four ARE motifs, which are located in the ULBP1-3'UTR, possess destabilizing function. Unfortunately, our attempts to discover specific microRNAs, which might contribute to regulation of ULBP1 gene expression, failed. However, according to the performed experiments we can not exclude, that microRNAs are involved in regulation of ULBP1 expression. Refinement of candidate microRNA selection and experimental approaches may help to elucidate the role of microRNAs in ULBP1 regulation.

Identification of microRNAs, which are functionally relevant in regulation of ULBP1 expression might allow to develop approaches to increase the susceptibility of cancer cells by microRNA-mediated upregulation of NKG2D-L. In a recent study, Mandelboim and colleagues could show, that transfection of antisense oligonucleotides against endogenous microRNAs into HFF lead to an induction of MICA and MICB protein expression and in consequence to enhanced killing of HFF by NK cells.[95] Experiments in mice and monkeys proved that it is possible to administer antisense oligonucleotides and specifically target endogenous microRNAs *in vivo*.[227-228] Although specific targeting of microRNAs is currently investigated only in preclinical studies, the development of new types of therapies to target microRNAs is an active field of research.[277]

V. RESULTS (PART 2)

1. Upregulation of NKG2D-L on human fibroblasts upon treatment with stress inducing reagents and histone deacetylase (HDAC) inhibitors

1.1 Results

Studies of Gasser and colleagues have revealed, that NKG2D-L expression can be induced on murine and human primary cell through treatment of these cells with reagents causing genotoxic stress and stalled DNA replication.[79] Based on these results, we treated HFF with ionizing irradiation (IR), 5-fluorouracil (5-FU) or aphidicolin (Table 13), conditions which have been reported to induce surface expression of ULBP1 on this cell type. ULBP1 and MICA/B surface expression was measured one day post treatment. Among these treatments, increased expression of ULBP1 was only observed for IR (MFIR(untreated) = 1.9±0.3 vs. MFIR(IR) = 3.3; Fig. 36). Also MICA/B was slightly upregulated upon IR treatment. Treatment with 5-FU and aphidicolin did not modulate the surface expression of neither ULBP1 nor MICA/B.

Since we have previously shown, that histone deacetylase inhibitors (HDACi) increase NKG2D-L levels and susceptibility of AML blasts to NK cell lysis (Diermayr et al. Blood 2008), HFF were also treated with valproic acid (VA) and trichostatin A (TSA) (Table 13). Valproic acid (VA) slightly increased the surface expression of ULBP1 (1.9±0.3 vs. 2.8) and MICA/B (1.4±0.2 vs. 2.8; Fig. 36). For treatment of HFF with TSA, a strong and reproducible induction of ULBP1 (1.9±0.3 vs. 4.1±0.8; $p = 0.0341$) and MICA/B (1.4±0.2 vs. 13.2±3.3; $p = 0.0081$) were observed. Therefore we conclude, that the HDACi TSA is the most potent treatment among the reagents that we tested to modulate NKG2D-L surface expression on HFF.

Table 13: Reagent for treatment of HFF

Reagent	Mode of action	Concentration
Ionizing irradiation (IR)	DNA damage	40 Gy
5-Fluorouracil (5-FU)	inhibition of DNA replication through incorporation into DNA and RNA	10 µM
Aphidicolin	inhibition of DNA polymerase	4 µM
Valproic acid (VA)	inhibition of histone deacetylase	1 mM
Trichistatin A (TSA)	inhibition of histone deacetylase	160 ng/ml

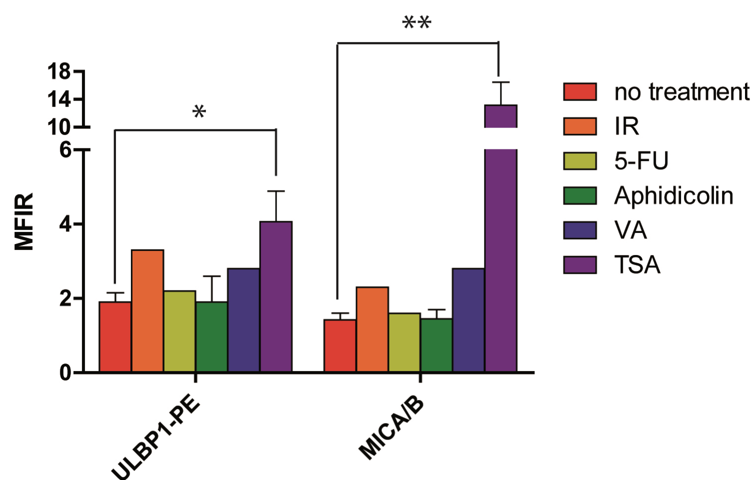

Figure 36: Upregulation of ULBP1 and MICA/B upon treatment in HFF with DNA damaging reagents and HDACi. ULBP1 and MICA/B surface expression one day after treatment with ionizing irradiation (IR), 5-fluorouracil (5-FU), aphidicolin, valproic acid (VA) and trichostatin A (TSA). Results are means ± SEM (n=1-4). * = $p < 0.05$; ** = $p < 0.01$.

To closer investigate the effect of TSA on the ULBP1 and MICA/B expression, we measured mRNA level in TSA treated HFF by qPCR. As already mentioned, surface expression of ULBP1 and MICA/B was highly induced upon TSA treatment (ULBP1: 2.1 fold; MICA/B: 7.2 fold; Fig. 37 A). Measurement of mRNA levels by qPCR revealed a 4 fold increase of ULBP1 transcript and a 7.2 fold increase of MICA transcript (Fig. 37 B). The stronger upregulation of ULBP1 transcript compared to protein might indicate that post-transcriptional mechanisms are involved in TSA-induced upregulation of ULBP1.

To investigate whether the upregulation of ULBP1 surface protein is mediated by the ULBP1-3'UTR, we examined the influence of TSA treatment on lentiviral luciferase reporter constructs. In HFF transduced with LV-RL-con, luciferase activity was 1.5 fold induced upon

treatment with TSA (Fig. 37C) suggesting that TSA influences the transcription by acting on the lentiviral hPGK promoter (Fig. 20B) though epigenetic changes. In HFF tranduced with LV-RL-U1-UTR, the luciferase activity was nearly restored upon treatment with TSA (4 fold induction compared to untreated cells; Fig. 37C), suggesting, that the ULBP1-3'UTR contributes to the observed upregulation of ULBP1. Therefore, not only transcriptional but also post-transcriptional events might participate in TSA-mediated induction of ULBP1.

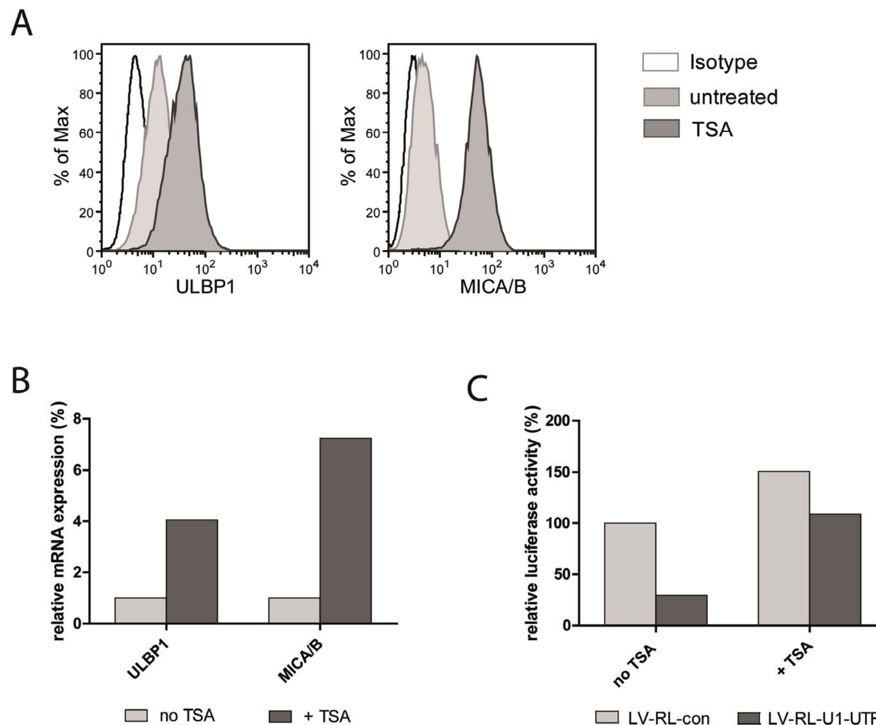

Figure 37: Effects of TSA treatment on ULBP1 regulation. (A) Measurement of ULBP1 and MICA/B surface expression on HFF one day after TSA treatment (dark grey) or without treatment (light grey) Isotype staining is indicated with black lined histogram. (B) Measurement of ULBP1 and MICA mRNA levels one day after TSA treatment (dark grey). Results are normalized to untreated counterparts (light grey). (C) The luciferase activity was measured upon transduction of LV-RL-con or LV-RL-U1-UTR into HFF, and subsequent treatment with TSA for one day. Luciferase activity was normalized against untreated cell, transduced with LV-RL-con.

1.2 Discussion

Investigation of leukemic blasts from patients with AML and of hematopoietic cancer cell lines have revealed, that many of these cells do not express NKG2D-L and therefore evade recognition and elimination by NK cells.[52,156] In order to make hematopoietic malignant cells vulnerable for NK cell attack, our aim was to find stimuli to upregulate these ligands on the cell surface, and to investigate the underlying regulation mechanisms. Previous studies have shown that NKG2D-L expression can be upregulated by stress-causing stimuli, e.g. oxidative stress, heat shock and irradiation, as well as by reagents causing DNA damage.[257] Therefore, we treated hematopoietic cell lines and primary bone marrow-derived CD34$^+$ stem cells with various chemotherapeutic reagents which are known to cause DNA damage or stalled DNA replication (e.g. 5-FU, methotrexate, endoxane, cytosar, adriblastin, aphidicolin) or with other conditions triggering cellular stress (e.g. heat shock, amino acid starvation, IR, oxidative stress). However, in our hand, none of these treatments lead to a distinct and reproducible induction of NKG2D-L surface expression on hematopoietic cancer cell lines and bone marrow CD34$^+$ stem cells (data not shown).

Also our attempts to reproduce published results failed. Previous studies have shown that treatment of murine or human fibroblasts with IR, 5-FU or aphidicolin, all stimuli which cause genotoxic stress, resulted in upregulation of ligands as consequence of activation of ATM, ATR and Chk2 kinases in the DNA damage response (DDR) pathway.[79] In contrast to the published results, treatment of the same type of HFF with 5-FU or aphidicolin did not or only marginally induced ULBP1 and MICA/B expression (Fig. 36) in our hands. A slight induction of ULBP1 on fibroblasts was only observed upon treatment of HFF with IR (MFIR(untreated) = 1.9±0.3 vs. MFIR(IR) = 3.3). It is difficult to explain these discrepancies to published data but to our knowledge, other groups also faced difficulties with reproducibility of these data (personal communications).

Our observation from experiments with malignant hematopoietic cells suggest, that the upregulation of NKG2D-L as a consequence of activation of the DDR pathway is most likely restricted to non-pathogenic cells or cells in early stages of tumor transformation. Inactivation of the DDR in later stages of carcinogenesis as consequence of genetic instability and accelerated tumor formation might be the reason, why malignant hematopoietic cells are not able to upregulate NKG2D-L upon genotoxic stress. Indeed, a study has shown that the DDR is highly activated in early urinary bladder tumor, whereas the activation is clearly reduced in

more advanced stages of tumorigenesis.[278] Furthermore, they showed that the activation of the ATM/ATR-Chk1/2-p53 cascade was frequently seen in absence of p53 mutations, suggesting that the latter event occurs later in tumorigenesis.

The observation that primary HFF in our hands did not respond to treatment with DNA damaging reagents might be explained by the fact, that long-term culturing of cells is per se a stress factor. Cultured cells might adapt to this stress factor and in consequence may inactivate the DDR pathway. Therefore, the induction of NKG2D-L in primary cells might be only successful, if these cells are only short-term cultured and if they did not undergo freezing and thawing (personal communications).

One explanation for the fact, that NKG2D-L are in general very difficult to induce may be, that NKG2D-L need to be tightly regulated and that special protection mechanisms exist in order to prevent unintended upregulation of ligands on healthy cells. Hematopoietic cells might require an even stronger protection than other cell types, since they are constantly exposed to metabolites, drugs, hormones and other substances in the peripheral blood or hypoxic conditions in the bone marrow. Therefore, these cells might have alternative stress sensors which ensure, that NKG2D-L are specifically upregulated only by highly threatening conditions.

In our earlier study we could show, that treatment of AML blasts with the HDACi valproic acid (VA) induced NKG2D-L expression and in consequence augmented recognition and killing of blasts by NK cells. In order to examine, if HDACi can also trigger NKG2D-L surface expression on HFF, we treated these cells with TSA and VA. TSA treatment resulted in a significant and reproducible upregulation of ULBP1 (MFIR(untreated) = 1.9±0.3 vs. MFIR(TSA) = 4.1±0.8) and MICA/B (1.4±0.2 vs. 13.2±3.3) (Fig 36). In contrast, treatment with VA affected the surface expression of the ligands only slightly. Examination of mRNA levels revealed a 4 fold and 7.2 fold increase of ULBP1 and MICA transcripts upon TSA treatment, respectively (Fig. 37). The stronger upregulation of ULBP1 transcripts compared to protein surface expression (4 fold vs. 2.1 fold) might indicate that post-transcriptional mechanisms are involved in TSA-induced upregulation of ULBP1. Indeed, TSA treatment of HFF transduced with the full-length luciferase vector resulted in a 4 fold induction of luciferase activity (Fig 37), which further argues for post-transcriptional regulation of ULBP1.

The molecular mechanisms of TSA-mediated upregulation of ULBP1 have not been elucidated so far. Recent studies revealed that treatment of cancer cells with HDACi

modulates the expression of microRNAs.[279-280] In order to examine, if these post-transcriptional regulators contribute to regulation of ULBP1 in response to HDACi, changes in microRNA levels upon treatment of HFF with TSA could be investigated by microRNA microarray or microRNA qPCR. Subsequent functional analysis of affected microRNAs might help to identify microRNAs which regulate ULBP1 expression.

Furthermore, this model could also be used to investigate transcriptional regulation mechanism of ULBP1, since TSA treatment significantly increased the ULBP1 mRNA level in HFF. Overexpression of HDAC is a common event in many cancer types and leads to formation of compact chromatin structures due to removal of acetyl groups from histones.[281] As consequence, transcription is impaired since transcription factors are unable to bind to promoter regions. Several reports showed, that MICA and MICB can be induced upon HDACi treatment. This induction has been linked to increased acetylation of histone H3 in the promoters of MICA and MICB.[282] Furthermore, upregulation of MICA/B upon HDACi treatment was dependent on the glycogen-synthase-kinase 3 (GSK3).[283] Our own studies showed, that not only MICA/B expression but also ULBP1 expression can be induced in primary AML blasts and HFF upon treatment with VA and TSA, respectively.[70] However the molecular mechanisms of HDACi-mediated upregulation of ULBP1 are largely unknown. Investigation of the acetylation status in the ULBP1 promoter region might help to elucidate the molecular mechanisms leading to ULBP1 induction. Upregulation of ULBP1 might also be caused by secondary effects upon TSA treatment. In order to identify molecular pathways, involved in TSA-mediated upregulation of ULBP1, inhibitors like caffeine and KU-55933[284] (inhibitor of ATM/ATR), rapamycin[285] (inhibitor of mTOR) or LY29402[285] (inhibitor of PI3K), all which have been used to investigate regulation of NKG2D-L expression by other groups, might help to address this question.

Multiple studies have shown that HDACi induce NKG2D-L expression selectively in cancer cells, while no increase of NKG2D-L was observed in non-malignant cells.[89,282,286] Therefore, pharmacological induction of NKG2D-L by treatment of patients with HDACi in combination with immunotherapeutical approaches, such as adoptive transfer of alloreactive NK cells, might represent a new therapeutic strategy in anticancer treatment.

2. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities

2.1 Publication

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NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities

Stefan Diermayr,[1] Heike Himmelreich,[1] Bojana Durovic,[1] Arina Mathys-Schneeberger,[1] Uwe Siegler,[1,2] Ulrich Langenkamp,[1] Jan Hofsteenge,[2] Alois Gratwohl,[3] André Tichelli,[3] Monika Paluszewska,[4] Wieslaw Wiktor-Jedrzejczak,[4] Christian P. Kalberer,[1] and Aleksandra Wodnar-Filipowicz[1]

[1]Department of Research, University Hospital Basel, Basel, Switzerland; [2]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland; [3]Stem Cell Transplant Team, University Hospital Basel, Basel, Switzerland; and [4]Department of Hematology and Oncology, Medical University of Warsaw, Warsaw, Poland

This study exploited alloreactivity of natural killer (NK) cells for augmenting the recognition of human acute myeloid leukemia (AML). To circumvent the inhibitory effect of killer immunoglobulin receptor (KIR) signaling, we generated NK-cell lines with single KIR specificities for major human leukocyte antigen (HLA) class I allotypes. We demonstrated efficient cytolysis of KIR-HLA class I–mismatched primary AML blasts even at low effector-to-target ratios. To define the impact of tumor-associated activating NKG2D-ligands (NKG2D-L), 66 AML patients at diagnosis were analyzed. NKG2D-L were selectively expressed on monoblastic cells in AML M4 and M5 yet absent or weakly expressed on myeloblastic cells in all AML subtypes. Paucity of cell-surface NKG2D-L was not the result of shedding because levels of soluble ULBP1 ligand measured in AML plasma were in the normal range. Notably, purified NKG2D-L$^+$ monoblastic cells were more susceptible to NK-mediated killing than NKG2D-L$^-$ myeloblastic cells. Accordingly, induction of cell-surface NKG2D-L by treatment with the histone deacetylase inhibitor, valproic acid, rendered cells more sensitive to NK cytolysis. These data suggest that adoptive transfer of selected populations of alloreactive HLA class I–mismatched NK cells in combination with pharmacologic induction of NKG2D-L merits clinical evaluation as novel approaches to immunotherapy of human AML. (Blood. 2008;111:1428-1436)

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Introduction

Improved outcome of acute myeloid leukemia (AML) after stem-cell transplantation across the human leukocyte antigen (HLA) class I barrier highlighted a potential of natural killer (NK) cells in recognition and elimination of residual malignant cells by the graft-versus-leukemia (GVL) effect in the absence of graft-versus-host disease (GVHD).[1,2] NK cells develop rapidly from transplanted progenitor cells but display numerous phenotypic abnormalities and a functional immaturity, which may limit their effectiveness in tumor rejection in vivo.[3,4] Adoptive transfer of mature NK cells in the early posttransplantation period is therefore a rational approach aimed at providing the patient with the benefit of competent cytotoxic effectors.[5]

NK cells are innate immunity CD56$^+$CD3$^-$ lymphocytes, the function of which is regulated by the integration of signals delivered from multiple inhibitory and activating receptors.[6] The killer immunoglobulin-like receptors (KIRs), which are clonally distributed in the NK-cell repertoire, recognize allelic groups of HLA class I molecules on target cells and are dominant determinants of NK-cell function.[7] NK cytotoxicity is inhibited on recognition of "self" HLA class I molecules, whereas absence or loss of HLA class I can render cells susceptible to NK-mediated lysis. In the transplant setting from haploidentical stem-cell donor, alloreactivity of NK cells is provided by the KIR-HLA class I mismatch.[8] In addition to sensing the missing self HLA class I molecules, NK cells need to be stimulated through engagement of activating NK receptors by specific cell-surface ligands expressed by target cells.[9,10] NKG2D is one of the best characterized receptors required for NK-mediated tumor immunosurveillance.[11] In mice, NKG2D function can protect the host from tumor initiation and also eradicate existing tumors expressing the H60 and Rae-1 ligands.[12,13] The human NKG2D ligands (NKG2D-L) include 2 families of proteins belonging to major histocompatibility complex class I (MIC)–related molecules, and UL-16 binding proteins (ULBP).[14,15] In epithelial tissue, the surface expression of NKG2D-L is up-regulated in response to cellular stress, including heat shock, DNA damage, and stalled DNA replication, which are common in human cancer.[16,17] A notion that NKG2D-L play a key role as tumor-associated ligands rendering cells susceptible to NK-mediated lysis, has encouraged pharmacologic approaches to achieve the NKG2D-L induction.[18,19]

Understanding the molecular interactions regulating the NK function has uncovered numerous escape mechanisms preventing recognition of leukemic blasts by NK cells. Unlike in solid tumors, which frequently down-regulate HLA class I molecules, malignant leukemic cells carry high levels of HLA class I having a protective effect.[20] NKG2D-L are absent or expressed at low levels in the majority of patients with acute leukemia,[21-23] thereby preventing efficient activating interactions. An additional route of tumor

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evasion from NK immunosurveillance is through proteolytic shedding of NKG2D-L molecules,[21,24] the soluble forms of which systemically down-regulate NKG2D receptor expression and lower the ability of NK cells to recognize tumor cells.[24,25] These immune escape mechanisms involving both inhibitory and activating receptor-ligand interactions may diminish the clinical GvL effect of NK cells used as cellular immunotherapy.

The aim of this study is to achieve efficient NK-mediated cytolysis of AML by integrating effects provided by the missing KIR-HLA class I interaction and by the NKG2D receptor-ligand recognition. We show that expression of NKG2D-L is dependent on the type of AML blasts and that pharmacologic up-regulation of NKG2D-L levels by the histone deacetylase (HDAC) inhibitor, valproic acid, leads to a significantly increased NK-mediated lysis of AML cells. The alloreactive effect of NK cells can be optimized by using NK-cell lines with single-KIR specificities mismatched with respect to HLA class I allotype of target tumor cells. Clinical implementation of these strategies may enhance the therapeutic impact of NK alloreactivity against human AML.

Methods

Leukemia patients and healthy donors

Patients presenting with primary AML (n = 55), secondary AML (n = 11), chronic myelomonocytic leukemia (CMML; n = 3), and healthy controls (n = 25) were included in the study. The diagnosis and definition of AML subtypes M1-M5 and CMML were based on morphologic, cytogenetic, and immunophenotypic criteria. The blast content was 59% plus or minus 3.5% (mean ± SEM). Peripheral blood samples from patients and healthy donor controls were collected in compliance with the guidelines of the Ethical Committees of the University Hospitals in Basel and Warsaw. Informed consent was obtained in accordance with the Declaration of Helsinki.

Cell lines

C1R-neo and C1R-ULBP1 transfectants[21] (kind gift of A. Steinle, Eberhard-Karls University Tübingen, Tübingen, Germany) were cultured in RPMI-1640 containing 10% fetal calf serum and 1 mg/mL Geneticin (all from Invitrogen, Carlsbad, CA). The lymphoblastic cell line 721.221 and 221-B*5801 (221-Bw4), 221-Cw*0304 (221-C1), and 221-Cw*0401 (221-C2) transfectants[26] (kind gift of P. Parham, Stanford University, Stanford, CA) were cultured as above, except that Geneticin was omitted.

Flow cytometry (fluorescence-activated cell sorter) and monoclonal antibodies

Fresh heparinized peripheral blood (50-200 μL) was stained with mouse monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein, or allophycocyanin (APC) followed by lysis of red blood cells with fluorescence-activated cell sorter (FACS) lysis buffer (BD Biosciences, San Jose, CA). Conjugated mAbs against human CD3, CD45, CD56, CD158b, and control mouse IgG1 (all from BD Biosciences), CD158a (Beckman Coulter, Marseille, France), and CD158e (BD Biosciences and R&D Systems, Abingdon, United Kingdom) were used. Staining with unconjugated mouse mAbs against ULBP1 (M295), ULBP2 (M311), ULBP3 (M551) (all at 10 μg/mL; kind gift of D. Cosman, Amgen, Seattle, WA), MICA/B and HLA class I (both at 10 μg/mL; BD Biosciences) was followed by staining with secondary goat α-mouse IgG-FITC (Jackson ImmunoResearch, West Grove, PA). At least 100 000 events were acquired using FACS (FACSCalibur; BD Biosciences), and analysis was performed using CellQuest Pro software (BD Biosciences). Expression level of NKG2D-L was defined as the mean fluorescence intensity ratio (MFI-R) of values obtained with specific mAbs divided by values given by secondary or control mAbs.

Purification and culture of AML cells

Mononuclear cells were isolated from peripheral blood by Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO) and cryopreserved until use. AML and CMML cell subpopulations were purified by sorting according to the side scatter and CD45 staining using FACSVantage (BD Biosciences). AML cells (5×10^6/mL) were cultured for 2 days in serum-free X-Vivo 10 medium (Cambrex, Verviers, Belgium) supplemented with cytokines, bovine serum albumin, insulin, and transferin, as described,[27] without and with valproic acid (VA) at 1 mM (Orfiril; Desitin, Liestal, Switzerland).

Purification and culture of NK cells

NK cells were obtained from peripheral blood mononuclear cells of a healthy donor (HLA A29/A23, B35/B44, Cw04/Cw12; KIR haplotype B) and purified by CD3$^+$ cell depletion followed by CD56$^+$ cell-selection with antibody-conjugated immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to a purity of more than 98%. Cells were stained with mAbs against CD158a-PE, CD158b-FITC, and CD158e-APC and single KIR-expressing NK cells were sorted using FACSVantage. Sorted cells were cultured in 24-well plates containing 2 mL of Iscove modified Dulbecco medium and 5% human AB$^+$ serum and supplemented with IL-2 (100 U/mL; kind gift of Novartis, Basel, Switzerland) and phytohemagglutinin (1 μg/mL, Murex Biotech, Dartford, United Kingdom) in the presence of irradiated allogeneic monononuclear cells.[28] After 500- to 1000-fold expansion during 14 to 21 days, the purity of NK cells was defined by staining with anti-CD56, -CD3, and respective -CD158 mAbs. NK-cell lines with the purity of more than 97% were cryopreserved.

Cellular cytotoxicity assays

The cytotoxic activity of NK-cell lines was tested by chromium release assay as described.[22] Briefly, 2×10^6 target cells were labeled with 250 μCi ^{51}Cr (Amersham, Little Chalfont, United Kingdom) in Iscove modified Dulbecco medium containing 10% fetal calf serum for 2 hours at 37°C, followed by incubation with NK cells for 4 hours at 37°C at the indicated effector-to-target (E/T) ratios. For blocking experiments, effector cells were preincubated with anti-NKG2D M585 mAb (kind gift of D. Cosman) at 10 μg/mL or mouse IgG1κ (BD Biosciences) for 1 hour at 37°C. Maximum ^{51}Cr release was determined with target cells lysed in 0.1% Triton-X. Percentage of cytotoxicity was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Experiments were performed in triplicate.

Analysis of soluble ULBP1

An ULBP1-specific enzyme-linked immunosorbent assay (ELISA) was developed to detect soluble ULBP1 (sULBP1) in plasma of AML patients and healthy blood donors. Plasma samples were diluted 1:1 with 50 mM Tris-HCL, pH 6.8, containing 1% Triton X-100 and incubated for 30 minutes at 37°C. Recombinant human (rh) ULBP1/Fc (R&D Systems) was used as standard. Supernatants and cell lysates prepared in 50 mM Tris-HCl, pH 8.0, containing 0.15% saponin and 1% Triton X-100,[29] from C1R-ULBP1 and C1R-neo cells were used as controls. ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with the capture anti-ULBP1 M295 mAb at 5 μg/mL in phosphate-buffered saline (PBS) overnight at 4°C, followed by blocking with ELISA buffer (2% bovine serum albumin, 0.2% Tween 20 in PBS) for 90 minutes at room temperature (RT) and washing. Samples were added in duplicates for 90 minutes at RT. After washing, biotinylated detection antibodies BAF1380 (R&D Systems) were added at 0.5 μg/mL in ELISA buffer for 1 hour at RT. ELISA was developed based on streptavidine alkaline phosphatase substrate system (Sigma-Aldrich), and the absorbance was measured at 405 nm with a microplate reader (Spectra MAX 190; Bucher Biotec, Basel, Switzerland). Detection range of ULBP1 was 0.2 to 25 ng/mL. For measurement of sMICA, we performed a sandwich ELISA using the capture anti-MICA M673 mAb (5 μg/mL, Amgen), the detecting biotinylated Ab BAF1300 (0.5 μg/mL, R&D Systems), and rh MICA/Fc (R&D Systems) as standard. Plasma samples were diluted in ELISA buffer. Detection range of sMICA was 0.2 to 50 ng/mL.

BLOOD, 1 FEBRUARY 2008 • VOLUME 111, NUMBER 3

Size exclusion chromatography was performed using a Superose 12-column (10 × 300 mm) equilibrated in PBS containing 250 mM NaCl, with 200 μL human plasma from a healthy peripheral blood donor containing 78 ng/mL sULBP1, as determined by ULBP1 ELISA. Fractions of 0.5 mL were collected in PBS containing 250 mM NaCl and tested in duplicate by ELISA for the presence of sULPB1. To determine the molecular weight of sULBP1, the column was calibrated with a set of molecular weight standards (6.5-63 kDa, Sigma-Aldrich; and Amersham, Freiburg, Germany).

Immunoprecipitation/Western analysis of ULBP1

Fractions 21 to 25 from the size exclusion chromatography, selected according to the ULBP1 ELISA, were pooled and subjected to immunoprecipitation with anti-ULBP1 M295 mAb, or mouse IgG1κ (BD Biosciences) or anti-NKG2D M585 mAb (10 μg each) as controls. Lysates from C1R-neo and C1R-ULBP1 transfected cells (1 mg protein) were subjected to immunoprecipitation with anti-ULBP1 M295 mAb (3.5 μg). Immunoprecipitates of the chromatography fractions and C1R cells were incubated with protein A-Sepharose 4B beads (Sigma, 50 μL/sample) for 4 hours at 4°C. For Western analysis, proteins were separated on sodium dodecyl sulfate-polyacrylamide gels (12%), blotted onto nitrocellulose, and ULBP1 was detected with biotinylated anti-ULBP1 BAF1380 (R&D Systems) Ab at 0.1 μg/mL. As a positive control rh ULBP1/Fc chimeric protein (3 ng) was loaded. Western blots were revealed with streptavidin-horseradish peroxidase (R&D Systems) and chemoluminescent substrate (SuperSignal; Pierce, Rockford, IL).

Statistical analysis

GraphPad software (San Diego, CA) and Student *t* tests were used to analyze NKG2D-L levels in AML and cytolysis of AML cells by NK cells.

Results

NKG2D ligands are preferentially expressed by monoblastic leukemic cells in AML M4 and M5

According to previous studies in AML, including our own, leukemic blasts from most patients have a NKG2D-L–negative/low phenotype, and sporadically, NKG2D-L–positive cases can be found.[21-23] To elucidate the pattern of NKG2D-L expression in AML, we performed a detailed FACS analysis of cell-surface ULBP and MIC ligands in 66 patients at diagnosis (Figure 1). The myeloid blast populations, analyzed according to side scatter and CD45 expression level, were further subdivided as myeloblastic CD45-dim (R1) and monoblastic CD45-intermediate (R2), which were clearly distinct from a population of CD45-bright residual normal lymphocytes (Figure 1A top panels). In AML M1-M3, in which the myeloblastic CD45-dim cells constitute the only blast population, NKG2D-L were absent or low (MFI-R of 1.0-2.0) in a majority of cases (ULBP1-negative M2 is shown in Figure 1A). In AML M4 and M5, containing CD45-dim and CD45-intermediate blast populations in different proportions, there was a marked difference in NKG2D-L expression dependent on the type of leukemic cells: myeloblastic cells were mostly ligand-negative, similar to M1-M3, whereas monoblastic cells were frequently ligand-positive (Figure 1A bottom panels). The results on ULBP1, ULBP2, ULBP3, and MICA/B expression are summarized in Figure 1B (details on NKG2D-L expression in 66 individual AML patients are given in Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article); an additional 3 patients with CMML were included in the study because of the presence of both myeloblastic and monoblastic cells. The myeloblastic cells displayed one or more NKG2D-L in only 11 of 69 (16%) patients and only at low levels (MFI-R up to 3.9). In contrast, the monoblastic cells expressed the ligands in 17 of 24 (71%) patients and at significantly higher levels (MFI-R up to 18.8; $P < .001$). This pattern indicates that NKG2D-L expression is related to the subtype of myeloid leukemia and that NKG2D-L surface density is higher on monoblastic cells that have undergone oncogenic transformation at later stages of myeloid differentiation.

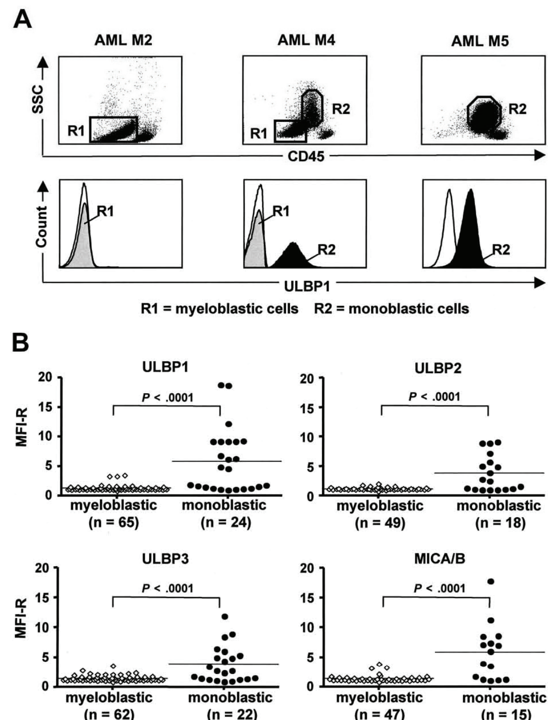

Figure 1. Expression of cell surface NKG2D-L by myeloblastic and monoblastic cells in AML and CMML. (A) FACS analysis of ULBP1 expression by AML peripheral blood cells of 3 patients with AML M2, M4, and M5. Gating of myeloblastic CD45-dim (R1) and monoblastic CD45-intermediate (R2) is shown in top panels, according to side scatter (SSC) and CD45 expression level. Histograms of ULBP1 expression by myeloblastic cells (R1, gray area) and monoblastic cells (R2, black area) are shown in bottom panels. (Thin line) Staining with secondary mAb. (B) Summary of ULBP1, ULBP2, ULBP3, and MICA/B expression levels, defined as MFI-R, by monoblastic and myeloblastic cells in all analyzed AML (n = 66) and CMML (n = 3) patients (for details on individual patients, see Table S1). Horizontal bars show the median values. ($P < .001$, significant difference in NKG2D-L expression levels by 2 types of leukemic blasts.)

Soluble ULBP1 is less abundant than soluble MICA in AML plasma

We considered the possibility that absence or low levels of membrane-bound NKG2D-L is the result of shedding from the cell surface. Proteolytic release of NKG2D-L has been described with MIC molecules, and recently with ULBP2, in various human tumors including hematopoietic malignancies.[21,30,31] Here, we established the ULBP1 ELISA to detect sULBP1 in human plasma (Figure 2). The specificity of the assay was demonstrated in control experiments in which high levels of ULBP1 were found in cell lysates and culture supernatants of C1R-ULBP1 transfectant cells, exceeding 200- to 300-fold the levels of endogenous ULBP1 in mock-transfected C1R-neo cells (Figure 2A). Using ULBP1-specific mAbs, the immunoprecipitation and Western analysis of cell lysates (Figure 2B) from C1R-ULBP1 transfectants revealed a

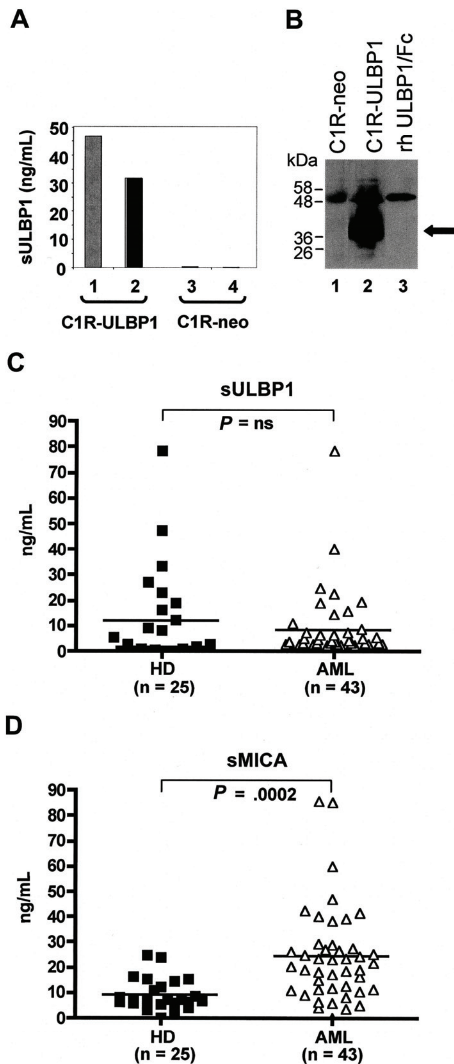

Figure 2. Identification of soluble ULBP1 (sULBP1) in AML plasma. (A) ULBP1 ELISA was established (see "Analysis of soluble ULBP1") and used to detect sULBP1 in C1R-ULBP1 cells (lane 1, culture supernatant gray bar; lane 2, cell lysate, black bar) but not in control C1R-neo cells (lanes 3 and 4). (B) Immunoprecipitation and Western analysis of sULBP1 in cell lysates of C1R-neo control cells (lane 1) C1R-ULBP1 cells (lane 2) and rh ULBP1/Fc (55 kDa; 3 ng, lane 3). Black arrow indicates the full-length ULBP1 molecule of approximately 40 kDa. (C,D) Levels of sULBP and sMICA in plasma from AML patients (n = 43; open symbols) and healthy donors (HD; n = 25; closed symbols), as determined by ELISA. Horizontal bars show the median values. Not significant (P = not significant) or highly significant ($P < .001$) difference in sULBP1 and sMICA levels in AML versus HD, respectively.

band of approximately 40 kDa, probably reflecting a full-length glycosylated ULBP1 molecule (lane 2), which was absent in C1R-neo control cells (lane 1). In human plasma, ULBP1 was found to exist as a 63 kDa complex, formed by 20 kDa portions corresponding to the ULBP1 extracellular domain (Figure S1).

Having established the specificity of ULBP1 ELISA, we assayed the levels of sULBP1 in plasma from AML patients (n = 43) compared with healthy donors (n = 25; Figure 2C). The average sULBP1 concentration in AML was 8.1 plus or minus 2.1 ng/mL, which was not significantly different from 11.6 plus or minus 3.7 ng/mL in control samples. In 9 of 43 AML patients, plasma levels of sULBP1 were above average values and reached 78.5 ng/mL, but similarly increased sULBP1 was found in 7 of 25 healthy donors. It should be noted that 9 samples with elevated sULBP1 included AML of different subtypes with $ULBP1^-$ and $ULBP1^+$ cell surface phenotypes, and there was no correlation to blast count. Next, we compared levels of sULBP1 with soluble MICA (sMICA; Figure 2D). Concentrations of sMICA in AML plasma were significantly higher than in plasma of healthy donors (24.5 ± 2.9 ng/mL vs 9.3 ± 1.2 ng/mL; $P < .001$), in agreement with previous studies.[21] In only one of 43 analyzed samples, both sULBP1 and sMICA levels were simultaneously exceeding the average values (details on sULBP1 and sMICA levels in individual AML plasma samples are given in Table S1). The lack of correlation between sULBP1 and sMICA levels suggests that different mechanisms are responsible for proteolytic cleavage of GPI-linked ULBP and transmembrane MIC ligands. Altogether, the measurement of plasma levels of sULBP1 demonstrated that ULBP1-negative/low phenotype of myeloblastic cells found in the majority of AML patients is not the result of ULBP1 shedding but represents an intrinsic property of AML blasts.

Generation of cytotoxic NK cells with single KIR-HLA class I specificity

Our next goal was to determine the relevance of cell surface NKG2D-L for recognition of AML blasts by NK cells. We took into account that AML blasts express HLA class I (see Table S1), and are therefore subject to dominant signals elicited by KIR-HLA class I interactions. To circumvent the inhibitory signaling which may mask the contribution of activating ligands, we generated NK-cell lines expressing single types of KIRs, each specific for one of the 3 major HLA class I allotypes. Healthy donor-derived purified $CD56^+CD3^-$ NK cells were stained with mAbs specific for CD158a (KIR2DL1) recognizing HLA-C group 2 alleles, CD158b (KIR2DL2 and KIR2DL3) recognizing HLA-C group 1 alleles, and CD158e (KIR3DL1) specific for HLA-Bw4 alleles.[32] The single KIR-expressing $CD158a^+$, $CD158b^+$, or $CD158e^+$ cells, termed subsequently "single-KIR" NK cells, represented small subsets of 14%, 5%, and 2% of donor NK cells, respectively (Figure 3A). The "single-KIR" subsets were purified by FACS sorting and cultured under conditions allowing up to 1000-fold expansion within 2 to 3 weeks[28] while maintaining the KIR specificity, thus generating NK-cell lines with 97% to 98% purity with respect to each type of KIR (Figure 3B). The functional specificity of these NK cells was tested against the HLA class I-negative lymphoblastoid 721.221 cell line stably transfected with genes encoding the HLA-Bw4 and HLA-C group 1 and group 2 alleles. $CD158a^+$, $CD158b^+$, and $CD158e^+$ NK-cell lines were highly cytotoxic against parental 721.221 cells, but not HLA class I transfectants matching the KIR specificity (Figure 3C). Subsequently, the activity of "single-KIR" NK-cell lines was tested against HLA class I-deficient leukemic K562 cell targets and against primary AML cells (Figure 3D). Using $CD158a^+$ and $CD158e^+$ cells as an example, we demonstrated that K562 cell lysis was independent of KIR specificity, whereas AML blasts missing the C2 group allele were selectively killed by mismatched $CD158a^+$ cells. These results confirmed the specificity of target cell recognition by "single-KIR" NK-cell lines.

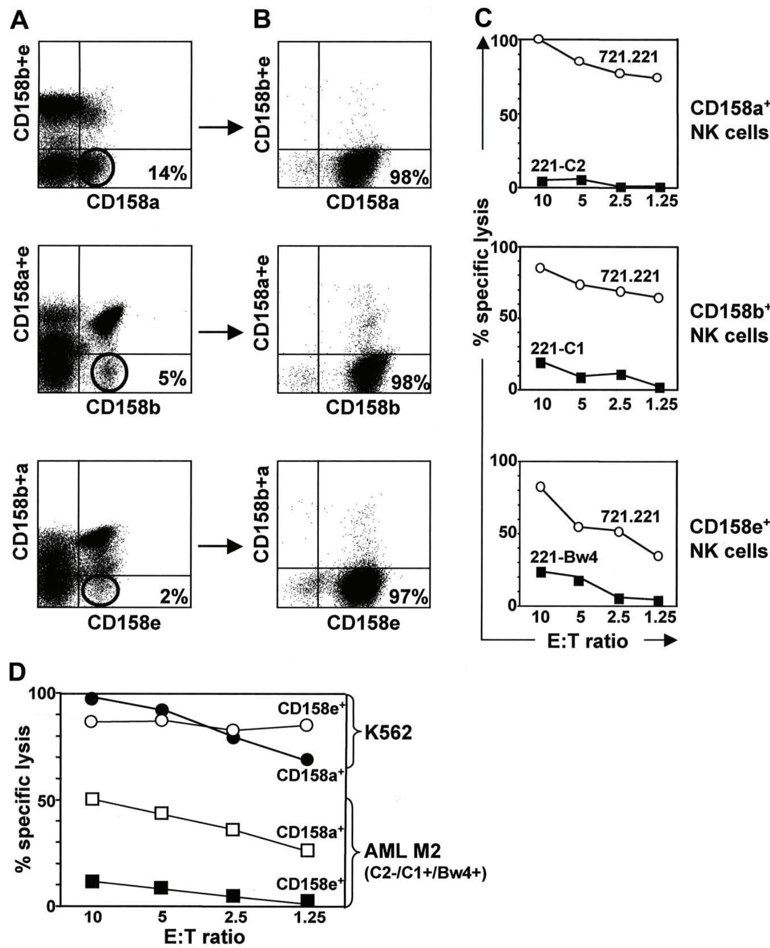

Figure 3. "Single-KIR" NK-cell lines specifically detect HLA class I allotypes. (A) FACS analysis of KIR (CD158a, 158b, and CD158e) expression by healthy donor-derived NK cells stained with a single anti-CD158 mAb (x-axis) versus a mixture of 2 anti-CD158 mAb (y-axis), as indicated. Single KIR-expressing CD158a$^+$, CD158b$^+$, and CD158e$^+$ NK-cell subpopulations of 14%, 5%, and 2%, respectively, are indicated. (B) FACS analysis of CD158a$^+$, CD158b$^+$, and CD158e$^+$ cells after FACS sorting and expansion in culture for up to 21 days revealed a purity of single KIR-expressing NK-cell lines of more than 97%. Numbers on plots are percentages of total CD56$^+$CD3$^-$ cells. (C) Cytotoxicity of CD158a$^+$, CD158b$^+$, and CD158e$^+$ NK-cell lines against 721.221 control cells (open symbols) and 221 transfectants with HLA class I allotypes belonging to group C1, C2, and Bw4 (closed symbols) at the indicated effector-to-target (E/T) ratios. (D) Cytotoxicity of CD158a$^+$ and CD158e$^+$ NK-cell lines against K562 cells (filled and open circles, respectively) and against leukemic blasts from a patient with AML M2 (filled and open squares, respectively). The HLA class I allotype of the patient is indicated.

"Single-KIR" NK-cell lines are highly cytotoxic against AML blasts

We examined the cytolytic effect of "single-KIR" NK-cell lines against leukemic blasts from 10 patients with various leukemia subtypes characterized by NKG2D-L$^-$ and NKG2D-L$^+$ phenotypes (Figure 4). Experiments were performed under defined KIR-HLA class I interactions by selecting both matched and mismatched NK-cell effectors according to HLA class I characteristics of each analyzed patient (for HLA class I haplotypes and predicted alloreactivity of NK cells, see Table S2). At the E/T ratio of 10:1, NK cells carrying mismatched KIRs were highly cytotoxic against leukemic blasts and their effect was significantly more pronounced than cytolysis observed with matched NK cells in case of every patient (Figure 4A). Remarkably, blasts were lysed by KIR-ligand mismatched NK cells even at the low E/T ratio of 1:1 (Figure 4B), underlining a strong cytolytic potential of "single-KIR" cell lines when selected according to HLA class I allotype of each target. There were large interindividual differences in susceptibility to alloreactive NK cells, obscuring the putative differences in lysis of NKG2D-L$^-$ and NKG2D-L$^+$ blasts (Figure 4A,B). Therefore, we purified NKG2D-L$^-$ myeloblastic cells and NKG2D-L$^+$ monoblastic cells (Figure 5A) and exposed the 2 types of blasts from patients with AML M5 and CMML to KIR-HLA class I-mismatched NK cells (Figure 5B). The lysis of NKG2D-L$^+$ monoblastic cells was significantly more efficient than NKG2D-L$^-$ myeloblastic cells over a wide range of E/T ratios ($P < .05$). This stronger killing of NKG2D-L$^+$ monoblastic cells was reduced to the level of killing of NKG2D-L$^-$ myeloblastic cells when NKG2D blocking mAbs were included in the assay (Figure 5B), indicating that expression of cell-surface NKG2D-L is relevant for blast recognition by alloreactive NK cells.

Valproic acid up-regulates NKG2D-L expression on leukemic blasts and enhances sensitivity to NK-mediated killing

The HDAC inhibitor VA is an antineoplastic drug promoting the myeloid differentiation of leukemic cells.[33] A previously reported induction of NKG2D-L in hepatoma cells exposed to VA[34] prompted us to investigate the consequences of VA treatment for the NKG2D-dependent recognition and killing of AML cells. The leukemic blasts from AML patients were treated for 2 days with VA, and the cell-surface expression of NKG2D-L and cytolytic effect of "single-KIR" NK-cell lines were examined (Figure 6). VA up-regulated the level of ULBP1 and MICA/B significantly stronger than observed by culturing cells in growth factor-containing medium only (Figure 6A). With 13 analyzed samples (which included 12 patients with NKG2D-L$^-$ AML M0-M2, and one patient with MICA/B$^+$ AML M4), the up-regulation of

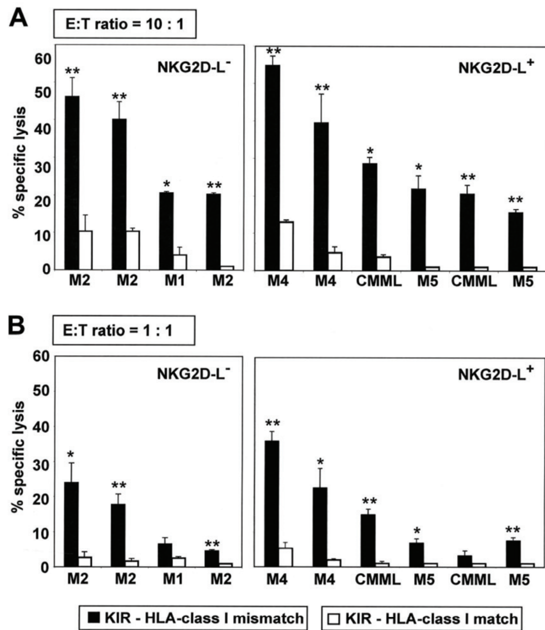

Figure 4. Cytotoxicity of "single-KIR" NK-cell lines against human primary leukemic cells. Cytolysis by CD158a⁺, CD158b⁺, and CD158e⁺ NK-cell lines was tested against cells from 10 AML and CMML patients, at the effector-to-target (E/T) ratios of 10:1 (A) and 1:1 (B). For each patient, NK-cell effectors were selected according to KIR-HLA class I mismatch (■) and KIR-HLA class I match (□), as specified in Table S2. Error bars represent SD. (*$P < .01$, **$P < .001$, significant difference between killing by KIR-HLA class I–mismatched and –matched NK cells.)

NKG2D-L in response to VA treatment was observed in case of 11 patients and reached 2.5 plus or minus 0.3-fold for ULBP1 ($P < .001$) and 2.9 plus or minus 0.5-fold for MICA/B ($P < .05$). The impact of VA-mediated increase of NKG2D-L levels was

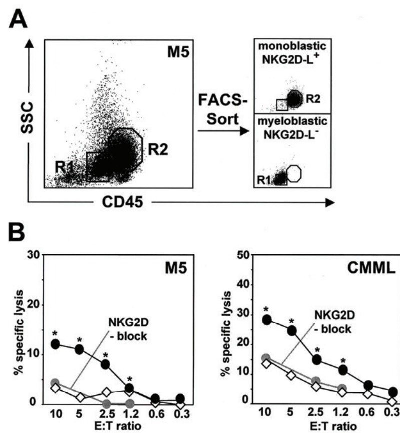

Figure 5. Susceptibility of NKG2D-L⁻ and NKG2D-L⁺ leukemic blasts to "single-KIR" NK-cell lines. (A) Myeloblastic CD45-dim (R1) and monoblastic CD45-intermediate (R2) cells from a patient with AML M5 were purified by FACS-sorting to obtain the NKG2D-L⁻ and NKG2D-L⁺ blasts (purity > 98%). The same purification procedure was applied to blasts from a patient with CMML (not shown). (B) Specific lysis of purified AML M5 and CMML patient-derived myeloblastic cells (◇) and monoblastic cells (●) by mismatched "single-KIR" NK-cell lines. Blocking α-NKG2D mAbs were preincubated with NK effectors before the cytotoxicity assay at the indicated E/T ratios (●). (* $P < .05$, significant difference between cytolysis of myeloblastic and monoblastic cells.)

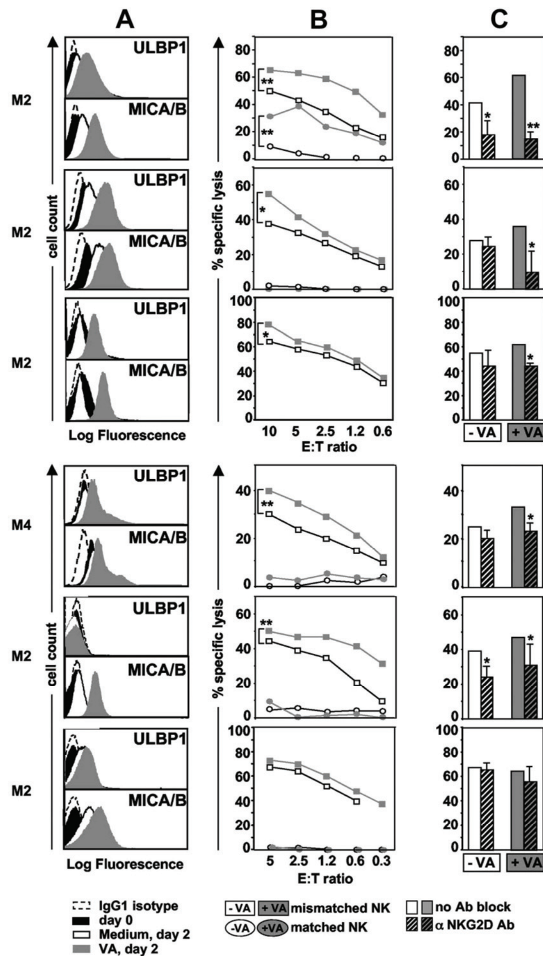

Figure 6. Increased susceptibility of leukemic blasts to "single-KIR" NK-cell lines after treatment with valproic acid (VA). (A) FACS analysis of ULBP1 and MICA/B expression levels by leukemic blasts from 6 AML patients untreated (black area), and after 2 days treatment with medium alone (black line) or VA (gray area); broken line, isotype-specific mAb staining. (B) Specific lysis of cells treated with medium alone (open symbols) and VA (filled symbols) by KIR-HLA class I–mismatched (squares) and matched NK-cell lines (circles). (*$P < .05$, **$P < .01$, significant difference between cytolysis of VA-treated and untreated AML blasts by NK cells.) (C) Reduction of specific lysis of AML cells by α-NKG2D blocking mAbs. KIR-HLA class I–mismatched NK cells were preincubated with blocking mAbs and used as effectors against AML cells cultured in medium without VA (□) and with VA (■). The effect of α-NKG2D mAbs is the average of results obtained at 3 NK cell/target cell ratios of 10:1 (or 5:1), 2.5:1, and 1.2:1. Lysis after use of α-NKG2D mAbs is shown as ▨. No blocking was observed with control IgG1 Abs (not shown). Error bars represent SD. (*$P < .05$, **$P < .01$, significant difference between lysis of VA-untreated and VA-treated AML blasts in the presence of α-NKG2D mAbs.)

tested in cytotoxicity assays performed under defined KIR-HLA class I interactions, specific for each patient (Figure 6B). The cytolysis of AML blasts by KIR-HLA class I–mismatched cells was enhanced after treatment with VA, and in one AML case (Figure 6B first middle panel), there was even a substantial induction of killing by matched NK cells. Addition of blocking anti-NKG2D mAbs partly abolished the cytolysis, and the blocking effect was stronger with VA-treated than VA-untreated cells (Figure 6C), indicating the contribution of NKG2D–NKG2D-L interactions to VA-modulated lysis of AML cells. These data demonstrated a role of HDAC inhibitors in enhancing the recognition and cytolysis of AML blasts by alloreactive NK cells.

Discussion

Relapse is a frequent severe complication in treatment of AML.[35] The clinical finding that HLA-nonidentical NK cells can exert the GvL effect and alter the outcome of bone marrow transplantation in AML opened a perspective of cellular immunotherapy with NK cells displaying alloreactivity toward the recipient's HLA type.[36,37] In this work, we demonstrate that the clinical impact of NK cells against human AML may be enhanced by selecting for KIR-HLA class I-mismatched subpopulations of NK cells and by combining the cellular therapy with a pharmacologic induction of NKG2D-L to augment the recognition of leukemic blasts by NK cells.

Alloreactivity of NK cells against tumors is based on recognition of tumor-associated activating ligands in the absence of inhibitory KIR engagement with HLA class I. Typically, however, only 2% to 20% of NK cells in peripheral blood carry a single type of a potentially alloreactive KIR.[1,23,38] Indeed, the size of a subset mismatched with respect to HLA class I allotype of the AML patient was shown to parallel the degree of NK-cell cytotoxicity against leukemic targets.[23] The low content of alloreactive NK cells may explain the requirement of high E/T ratios of up to 100:1 to achieve a pronounced killing of primary AML in vitro.[21-23,39] We demonstrated the possibility of generating NK-cell lines expressing a single type of KIR, with CD158a$^+$, CD158b$^+$, or CD158e$^+$ phenotypes, which can be selectively used according to a missing HLA class I allotype on target cells. In such a mismatched setting, every tested AML sample was lysed with a significantly higher efficiency than by NK cells carrying KIRs, which matched the patient's HLA class I. Consequently, prominent cytolysis was achieved using 10:1 and even 1:1 proportion of alloreactive NK cells toward AML targets. Selection for and appropriate choice of "single-KIR" cells eliminates not only nonalloreactive NK-cell subsets, but also hyporesponsive KIR-negative cells,[40] from the population of NK effectors. Use of selected competent "single-KIR" NK lines represents an alternative to the postulated use of blocking antibodies against KIRs[41,42] to attenuate inhibitory KIR signals. Interestingly, in a mouse model of pulmonary tumors, the tumor load was significantly reduced by a single injection of inhibitory Ly49 ligand-mismatched, but not matched, NK cells, providing evidence for the in vivo effectiveness of alloreactive NK cells with a single inhibitory receptor–ligand incompatibility.[43] Although 20% to 60% of "single-KIR" NK-cell lines expressed the inhibitory receptor NKG2A specific for HLA-E,[44] elimination of NKG2A-carrying cells to further enhance the lytic potential of alloreactive cells is of no benefit because the NKG2A$^-$ phenotype is not stable and selected NKG2A$^-$ cells reacquire the receptor on culture (data not shown).

Importantly, after FACS-purification of CD158a$^+$, CD158b$^+$, and CD158e$^+$"single-KIR" NK-cell subsets, we showed an efficient, up to 1000-fold, cell expansion without loss of KIR specificity or expression level in the resulting NK-cell lines. Cytokine-dependent expansion of NK cells is associated with a significant up-regulation of the cell surface expression of the activating receptor NKG2D, which plays an important role in triggering the NK cell–mediated tumor cell lysis.[45-47] Accordingly, knocking-down of NKG2D in human NK cells virtually abolishes the killing of leukemic targets.[48] The "single-KIR" NK-cell lines, which were cultured in the presence of IL-2, harbored high levels of NKG2D (MFI-R of approximately 100), possibly strengthening the NK cell–target cell recognition. The PHA and cytokine stimulation accompanying the ex vivo expansion is essential for effective alloreactivity because resting "single-KIR" NK cells are poor antileukemic effectors even in the presence of KIR-HLA class I mismatch (data not shown).

Ligands belonging to the ULBP and MIC families are serving as tumor-specific antigens for recognition and destruction in a NKG2D-receptor dependent process. As a newly recognized mechanism of innate immunity, induction of this ligand system represents a response to genomic DNA damage as means to eliminate the precancerous and cancer cells.[49] In AML, however, the NKG2D-L$^+$ cases were found only sporadically.[21-23] By performing a detailed phenotypic analysis of specific subpopulations of AML blasts, we now show that myeloblastic CD45-dim cells are NKG2D-L$^-$/low in all patients, whereas monoblastic CD45-intermediate cells frequently express high ligand levels. This pattern of ligand distribution indicates that expression of NKG2D-L is preferentially clustered in leukemias of the M4 and M5 subtypes, and provides a strong support to our earlier conclusion that NKG2D-L are acquired at late maturation stages in the myeloid lineage differentiation process.[22] This model is reinforced by our current finding that paucity of cell surface ULBP1 was not the result of shedding. Unlike elevated sMICA in AML, sULBP1 levels in AML were high in only a minority of patients, without a clear prevalence according to AML subtype. This pattern is reminiscent of sporadically elevated soluble ULBP2 described recently in sera of leukemic patients and healthy donors.[31] The susceptibility of AML to cytotoxic elicited by alloreactive NK cells was highly heterogeneous, and the extent of lysis of individual AML patient-derived blasts did not reflect the cell-surface NKG2D-L levels. This is likely the result of contribution of ligands for other important activating NK-cell receptors, such as molecules interacting with the natural cytotoxicity receptors[22] and ligands for DNAM-1.[23] These ligand families may also contribute to a low but consistently observed susceptibility of AML to cytolysis by KIR-HLA class I–matched NK-cell lines.

By FACS-based purification of NKG2D-L$^-$ myeloblastic cells and NKG2D-L$^+$ monoblastic cells, we showed that NKG2D-L$^+$ cells were significantly more susceptible to NK-mediated killing than NKG2D-L$^-$ cells, thus demonstrating the importance of cell-surface NKG2D-L for recognition and killing of leukemic targets. Consequently, we took a pharmacologic approach to achieve the NKG2D-L up-regulation on NKG2D-L$^-$/low AML blasts. Recently, we demonstrated that differentiating drugs, including the HDAC inhibitor trichostatin A, reversing the epigenetic silencing mechanisms can up-regulate the NKG2D-L in the myeloid leukemia cell line HL-60.[19] Here we used VA, reported recently as potent inducer of NKG2D ligands in hepatocytes,[34] and undergoing clinical trials in myeloid malignancies.[33,50] Remarkably, treatment of AML cells with VA produced a significant increase of NKG2D-L levels, resulting in enhanced susceptibility to NKG2D-mediated killing. Of particular importance is that a majority of analyzed AML samples with VA-induced changes belonged to NKG2D-L$^-$ AML M0-M2. It is also noteworthy that treatment with VA in some cases even enhanced the killing of blasts by HLA class I–matched NK cells, indicating that activating signals may override the negative KIR signaling, as described with murine NKG2D-L–overexpressing tumor targets.[51] According to our preliminary data, VA treatment also has a stimulatory effect on expression level of DNAM-1 ligands on AML cells. These data indicate that VA mediates specific sensitization of malignant AML cells for immune

effector mechanisms and may represent a valuable treatment combination in the setting of KIR-HLA class I incompatibility. Based on our in vitro analyses, the pharmacologic application of VA is not likely to inhibit the cytotoxic properties of NK cells but may be associated with inhibitory effect on expansion of adoptively transferred NK cells (data not shown).

Potential clinical benefits of NK cells from HLA-nonidentical donors include not only GvL reactivity but also prevention of GvHD through elimination of dendritic cells.[52] Indeed, in a mouse allogeneic transplant model, adoptive transfer of alloreactive NK cells reduced the tumor load along with reducing the GvHD.[43] In clinical transplantation, the KIR-HLA class I mismatches may not protect from GvHD when NK cells are derived from unrelated stem-cell donors,[53] whereas they may reduce the risk of GvHD in T-cell–depleted haploidentical stem cell transplants.[54] Infusions of donor-derived haploidentical NK cells have recently been initiated and proven as safe and of potential clinical benefit in some patients.[55,56] Our data indicate that adoptive transfer of haploidentical donor-derived NK cells should be preceded by selection of cells alloreactive toward the recipient's HLA. Subsequent IL-2-driven ex vivo expansion of selected NK cells will yield activated effectors in high numbers to reach effective proportions to the tumor targets in vivo. Use of selected cell subsets will allow reduction of the culture volume during cell expansion and, furthermore, will avoid infusion of large quantities of nonalloreactive bystander NK cells. We propose that appropriate use of alloreactive NK cells in combination with pharmacologic induction of NKG2D-L merits clinical evaluation as novel approaches to prevent relapses of human AML with NK-cell immunotherapy.

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Authorship

Contribution: S.D. designed and performed research, analyzed data, and edited the paper; H.H., B.D., A.M.-S., U.S., and U.L. designed and performed research; J.H. designed research and analyzed data; A.G., A.T., M.P., and W.W.-J. provided clinical data; and C.P.K. and A.W.-F. designed research, analyzed data, and wrote and edited the paper.

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Correspondence: Aleksandra Wodnar-Filipowicz, Department of Research, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland; e-mail: Aleksandra.Wodnar-Filipowicz@unibas.ch.

References

1. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood. 1999;94:333-339.
2. Velardi A, Ruggeri L, Alessandro Moretta Moretta L. NK cells: a lesson from mismatched hematopoietic transplantation. Trends Immunol. 2002;23:438-444.
3. Chklovskaia E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: effects of endogenous flt3 ligand. Blood. 2004;103:3860-3868.
4. Cooley S, Xiao F, Pitt M, et al. A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self MHC is developmentally immature. Blood. 2007;110:578-586.
5. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol. 2007;7:329-339.
6. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
7. Parham P. Taking license with natural killer cell maturation and repertoire development. Immunol Rev. 2006;214:155-160.
8. Ruggeri L, Capanni M, Mancusi A, Martelli MF, Velardi A. The impact of donor natural killer cell alloreactivity on allogeneic hematopoietic transplantation. Transpl Immunol. 2005;14:203-206.
9. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.
10. Lanier LL. On guard-activating NK cell receptors. Nat Immunol. 2001;2:23-27.
11. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3:781-790.
12. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A. 2001;98:11521-11526.
13. Smyth MJ, Swann J, Cretney E, Zerafa N, Yokoyama WM, Hayakawa Y. NKG2D function protects the host from tumor initiation. J Exp Med. 2005;202:583-588.
14. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285:727-729.
15. Cosman D, Mullberg J, Sutherland CL, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 2001;14:123-133.
16. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A. 1996;93:12445-12450.
17. Gasser S, Orsulic S, Brown EJ, Raulet DH. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature. 2005;436:1186-1190.
18. Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, Odum N. Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. Cancer Res. 2005;65:11136-11145.
19. Rohner A, Langenkamp U, Siegler U, Kalberer CP, Wodnar-Filipowicz A. Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemia cells to natural killer cell-mediated lysis. Leuk Res. 2007;31:1393-1402.
20. Brouwer RE, van der Heiden P, Schreuder GM, et al. Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. Hum Immunol. 2002;63:200-210.
21. Salih HR, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood. 2003;102:1389-1396.
22. Nowbakht P, Ionescu MC, Rohner A, et al. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood. 2005;105:3615-3622.
23. Pende D, Spaggiari GM, Marcenaro S, et al. Analysis of the receptor–ligand interactions in the natural killer-mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence for the involvement of the Poliovirus receptor (CD155) and Nectin-2 (CD112). Blood. 2005;105:2066-2073.
24. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature. 2002;419:734-738.
25. Wiemann K, Mittrucker HW, Feger U, et al. Systemic NKG2D down-regulation impairs NK and CD8 T cell responses in vivo. J Immunol. 2005;175:720-729.
26. Draghi M, Yawata N, Gleimer M, Yawata M, Valiante NM, Parham P. Single-cell analysis of the human NK cell response to missing self and its inhibition by HLA class I. Blood. 2005;105:2028-2035.
27. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med. 1997;186:619-624.
28. Siegler U, Kalberer CP, Nowbakht P, Sendelov S, Meyer-Monard S, Wodnar-Filipowicz A. Activated

natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. Leukemia. 2005;19:2215-2222.

29. Bumgarner GW, Zampell JC, Nagarajan S, et al. Modified cell ELISA to determine the solubilization of cell surface proteins: applications in GPI-anchored protein purification. J Biochem Biophys Methods. 2005;64:99-109.

30. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol. 2002;169: 4098-4102.

31. Waldhauer I, Steinle A. Proteolytic release of soluble UL16-binding protein 2 from tumor cells. Cancer Res. 2006;66:2520-2526.

32. Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. Annu Rev Immunol. 2002;20:217-251.

33. Cimino G, Lo-Coco F, Fenu S, et al. Sequential valproic acid/all-trans retinoic acid treatment reprograms differentiation in refractory and high-risk acute myeloid leukemia. Cancer Res. 2006; 66:8903-8911.

34. Armeanu S, Bitzer M, Lauer UM, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. Cancer Res. 2005;65:6321-6329.

35. Burnett AK. Current controversies: which patients with acute myeloid leukaemia should receive a bone marrow transplantation? An adult treater's view. Br J Haematol. 2002;118:357-364.

36. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-vs-leukemia effect. Blood. 2002;100:1935-1947.

37. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, Velardi A. Natural killer cell alloreactivity in allogeneic hematopoietic transplantation. Curr Opin Oncol. 2007;19:142-147.

38. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. J Immunol. 2004;172:644-650.

39. Costello RT, Sivori S, Marcenaro E, et al. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood. 2002;99:3661-3667.

40. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity. 2006;25:331-342.

41. Sheridan C. First-in class cancer therapeutic to stimulate natural killer cells. Nat Biotechnol. 2006;24:597.

42. Koh CY, Blazar BR, George T, et al. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. Blood. 2001; 97:3132-3137.

43. Lundqvist A, McCoy JP, Samsel L, Childs R. Reduction of GVHD and enhanced antitumor effects after adoptive infusion of alloreactive Ly49-mismatched NK cells from MHC-matched donors. Blood. 2007;109:3603-3606.

44. Nguyen S, Dhedin N, Vernant JP, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood. 2005;105:4135-4142.

45. Pende D, Cantoni C, Rivera P, et al. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of non-epithelial origin. Eur J Immunol. 2001;31:1076-1086.

46. Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J Immunol. 2002;168: 671-679.

47. Sutherland CL, Rabinovich B, Chalupny NJ, Brawand P, Miller R, Cosman D. ULBPs, human ligands of the NKG2D receptor, stimulate tumor immunity with enhancement by IL-15. Blood. 2006;108:1313-1319.

48. Baeriswyl V, Wodnar-Filipowicz A, Kalberer CP. The effect of silencing NKG2D through RNA interference on receptor functions in interleukin-2-activated human natural killer cells. Haematologica. 2006;91:1538-1541.

49. Gasser S, Raulet DH. The DNA damage response arouses the immune system. Cancer Res. 2006;66:3959-3962.

50. Bug G, Ritter M, Wassmann B, et al. Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer. 2005;104:2717-2725.

51. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature. 2001;413: 165-171.

52. Moretta L, Ferlazzo G, Mingari MC, Melioli G, Moretta A. Update on natural killer cells: cross-talk with dendritic cells and role in the cure of acute myeloid leukemias. Cancer J. 2003;9:232-237.

53. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-vs-host disease (GVHD) following unrelated donor allogeneic HCT. Blood. 2007; 109:5058-5061.

54. Ruggeri L, Mancusi A, Capanni M, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood. 2007;110:433-440.

55. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia. 2004;18:1835-1838.

56. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-3057.

2.2 Supplementary Data

Table 14: Ligands on myeloblastic/monoblastic cells and in plasma ofAML (n=66) and CMML (n=3)

	% blasts[a]			ULBP1[b]		ULBP2[b]		ULBP3[b]		MICA/B[b]		HLA-class I[b]		sNKG2D-L[c]	
2°AML	total	myelo-	mono-	myelo-	mono-	myelo-	mono-	myelo-	mono-	myelo-	mono-	myelo-	mono-	sULBP1	sMICA
AML1	50	50	0	1.5	na			2.8	na					24.9	0.19
AML2	3	3	0	1.6	na			3.6	na					0.1	16.45
AML3	50	50	0	1.4	na			1.3	na	1.8	na	129	na	5.31	5.78
AML4	40	40	0	1.1	na			1.2	na					0.1	85.71
AML5	30	30	0	1.1	na									3.5	23.43
AML6	80	80	0	1.4	na	1.2	na	2.1	na	1.6	na			8.5	20.33
AML7	20	20	0	1.2	na	1.2	na	1.3	na			314.1	na	3.23	21.58
AML8	35	35	0	1.8	na	1.4	na	1.6	na	1.6	na	128.5	na	4.13	4.88
AML9	50	50	0	1.6	na	1.4	na	1.7	na	1.4	na	57.9	na	3.1	26.52
AML10	25	25	0	1.1	na	1.2	na	1.3	na	1.3	na	270.7	na		
AML11	5	5	0	1.1	na	1.1	na	2	na	1.1	na	120	na		
M1															
AML12	80	80	0	1.5	na			2.4	na			62.6	na	0.1	19.13
AML13	10	10	0	1.2	na			2.1	na					0.1	12.51
AML14	15	15	0	1.5	na	1	na	1.2	na	1.4	na	144.2	na	18.97	17.38
AML15	90	90	0	1.2	na	1	na	1.2	na	1.2	na	49.6	na	7.23	23.51
AML16	40	40	0	1	na	1	na	1.2	na	1	na	32.2	na	2.47	42.23
AML17	86	86	0	1.2	na	1.1	na	1.2	na	1.2	na	87.5	na	6.42	29.47
AML18	95	95	0	1.4	na			1.7	na	1.6	na	74.6	na		
AML19	80	80	0	1.1	na	1.1	na	1.1	na	1.3	na	81.3	na		
M2															
AML20	75	75	0	1.1	na			1.1	na						
AML21	35	35	0	1.3	na			1.4	na			29.2	na		
AML22	70	70	0	1.2	na			2.1	na			164.1	na	0.1	6.54
AML23	55	55	0	1.3	na	1.2	na	1.3	na					3.4	18.67
AML24	45	45	0	1	na	1	na	1.7	na	1.1	na	108.7	na	2.91	11.23
AML25	70	70	0	1	na	1.1	na	1.5	na	1	na	166.4	na		
AML26	50	50	0	1.1	na	1.1	na	2.3	na	1.2	na	168.1	na		
AML27	40	40	0	0.8	na	0.7	na	0.8	na	0.8	na	164.8	na		
AML28	65	65	0	1	na	0.9	na	1	na	1	na	168.8	na	19.24	11.1
AML29	6	6	0	1.6	na	1.1	na	1.1	na	1.6	na	31.8	na		
AML30	30	30	0	3.5	na	2	na	2.3	na	3.9	na	129.4	na	4.8	8.26
AML31	70	70	0	1	na	1	na	1.2	na	1.1	na	34.2	na	3.39	24.59
AML32	5	5	0	1.4	na	1.6	na	1.5	na	1.8	na	65.5	na	5.11	23.03
AML33	90	90	0	1.1	na	1.1	na	1.1	na	1.1	na	82.7	na	2.92	18.77
AML34	80	80	0	1.2	na	1.1	na	1.2	na	1.3	na	189.2	na	6.23	59.89
AML35	35	35	0	1.2	na	1.1	na	1.4	na	1.5	na	84.4	na	4.37	26.22
AML36	10	10	0	1	na	1.1	na	1.3	na	1.1	na	287.5	na		
AML37	90	90	0	1.2	na	1.2	na	1.3	na	1.5	na	53.6	na		
AML38	75	75	0	1	na	1	na	0.9	na	1	na	301.9	na		
AML39	90	90	0	1.2	na	1.2	na			1.1	na	99.2	na		
M3															
AML40	95	95	0	1.1	na	1.1	na	1.1	na						
AML41	40	40	0	1.5	na	1.1	na	1.6	na	1.3	na	20.4	na	2.94	17.93
AML42	45	45	0	1.2	na	1.2	na	1.3	na	1.2	na	175.2	na		
AML43	95	95	0	1	na	1	na	1	na	1	na	14.5	na	3.76	46.98
AML44	70	70	0	3.3	na	1.4	na	1.8	na	2.2	na	83.4	na	3.24	84.91
AML45	95	95	0	1.1	na	1.1	na	1	na	1.2	na	70.7	na	2.92	26.85
M4															
AML46	80	60	20	1	1.2	1	1.2	1.1	1.6						
AML47	90	60	30	1.2	6.8			1.7	5.4						
AML48	40	25	15	1.2	1.8			1.2	2.8			121.2	108.5	0.1	3.5
AML49	85	40	45	1.1	1.7			1.2	1.4			29.4	18.9	78.52	4.05
AML50	95	20	75	1	1.1			1.1	2.4	1.1	1.3	45.9	33.6	0.1	40.2
AML51	45	5	40	1.7	18.7	1.5	8.9	1.5	8.5			90.8	63.8	7.22	27.67
AML52	70	10	60	1.1	9.2	1.1	5	1.1	6.1	1.1	8.7	203.6	156.1	2.92	11.77
AML53	90	75	15	1	1	1	1	1.2	1.1	1	1.2	34.2	12.8	22.38	39.08
AML54	85	70	15	1.1	6.3	1	2.8	1.3	2.8	1	4	39.4	44.9	40.27	10.49
AML55	95	75	20	1.2	9.2	1.2	5.7	1.2	6.5	1.3	7.5	60.1	67.7	2.51	38.46
AML56	90	70	20	1.3	9.2	1.1	4.9	1.1	4.9	1.2	7.1	69.7	86.7		
AML57	20	10	10	3.3	1.3	1.8	1	1.3	0.9	3.3	1.3	10.4	10	3.3	24.64
AML58	80	40	40	1.2	1.9	1.1	1	1.2	1.3	1.9	3.6	28.5	27.1		
M5															
AML59	80	40	40	1.2	12.2	1	7.2	1.2	5						
AML60	10	0	10	na	1.6	na	1.6	na	1.5						
AML61	95	0	95	na	4.6	na	2.5	na	3.5			na	98.9	15.69	14.79
AML62	40	0	40	na	1.5	na	1.3	na	1.8			na	93.7		
AML63	85	0	85	na	6.2					na	6.1	na	304.8		
AML64	60	10	50	1	4.9	1.3	3.9	1.4	4.4	1.2	7.2	91	39.4	2.83	28.99
AML65	90	20	70	1	1.1	1	1.1	1	1	1	1.1	114	99.6	10.83	8.89
AML66	80	10	70	1.4	9.3	1.6	8.9	2.1	9	3.2	11.3	137.9	116		
CMML															
CMML1	80	50	30	1.3	9.2					1.4	8.6	88.5		2.37	41.57
CMML2	40	10	30	1.1	18.8	1.2	9.1	1.6	11.9	1.1	17.8	56.7		4.11	25.23
CMML3	30	10	20	1	1.3	1	1.1	1.3	1.3	1.1	1.8	239.7	106.1		
Mean	**58.9**	**45.2**	**13.7**	**1.3**	**5.8**	**1.2**	**3.8**	**1.5**	**3.9**	**1.4**	**5.9**	**107.7**	**82.7**	**8.1**	**24.5**
n	69	69	69	65	24	49	18	62	22	47	15	53	18	43	43
SEM	3.5	3.7	2.9	0.1	1.1	0.0	0.7	0.1	0.6	0.1	1.2	10.4	16.3	2.1	2.9

[a], % of blasts in peripheral blood; [b], MFI-R; [c], ng/mL; na, not applicable; 2^0 AML, secondary AML; SEM, standart error of the mean.

Table 15: HLA-class I haplotype of patients and potential NK cell recognition by KIR-HLA class I mismatch

Patient Diagnosis*	HLA-A	HLA-B	HLA-C	Missing HLA-class I	Mismatched KIR (CD158)
M2	A02, A32	B07, B15 (Bw4, Bw6)	Cw07, Cw07 (C1)	C2	a
M2	A01, A03	B08, B35 (Bw6)	Cw04, Cw07 (C1, C2)	Bw4	e
M1	A02, A24	B51, B55 (Bw4, Bw6)	Cw03, Cw12 (C1)	C2	a
M2	A23, A30	B07, B58 (Bw4, Bw6)	Cw07, Cw07 (C1)	C2	a
M4	nd	B35, B35 (Bw6)	Cw04, Cw04 (C2)	Bw4, C1	b, e
M4	A24, A29	B18, B44 (Bw4, Bw6)	Cw12, Cw16 (C1)	C2	a
CMML	A02, A03	B51, B44 (Bw4)	Cw05, Cw15 (C2)	C1	b
M5	A03, A29	B07, B35 (Bw6)	Cw04, Cw07 (C1, C2)	Bw4	e
CMML	A01, A11	B08, B60 (Bw6)	Cw03, Cw07 (C1)	Bw4, C1	b, e
M5	A03, A29	B38, B44 (Bw4)	Cw16, Cw16 (C1)	C2	a

* the order of samples corresponds to Figure 4; nd, not determined.

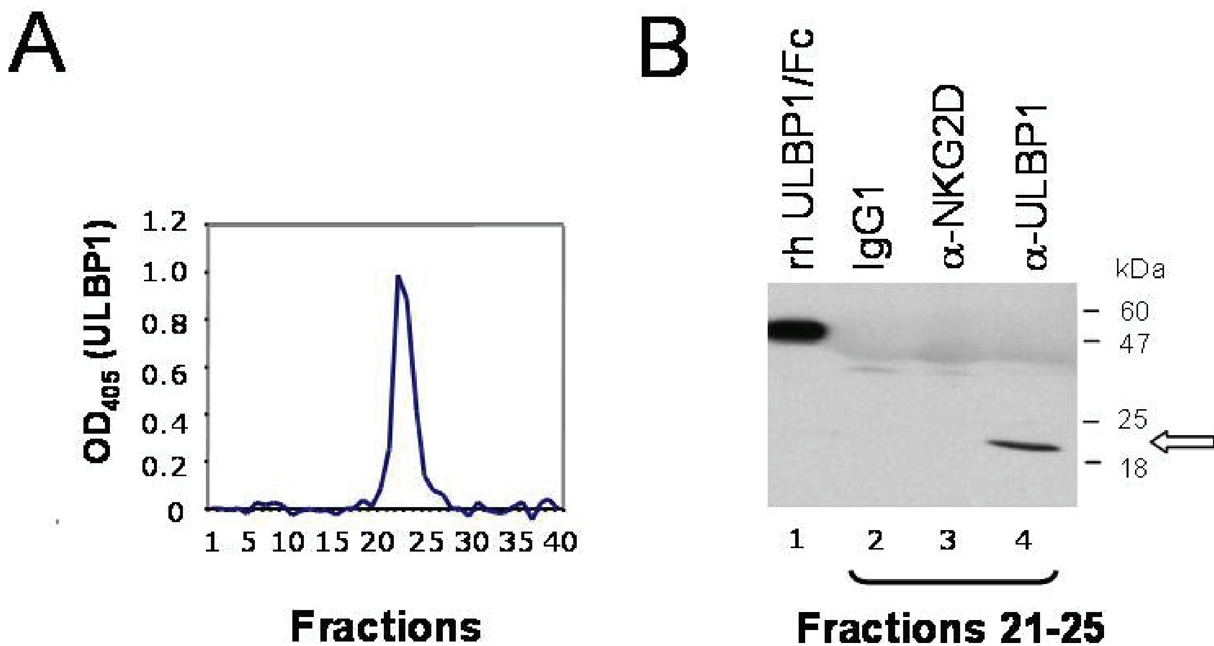

Figure 38: (A) A biochemical ULBP1 detection approach in which human plasma was subjected to fractionation by size exclusion chromatography followed by ELISA analysis of the eluate (see Methods). ULBP1 eluted as a single peak with an estimated molecular weight of 63 kDa. (B) The ULBP1-containing fractions (21-25) were pooled and subjected to immunoprecipitation and Western blot analysis. ULBP1-specific mAbs immunoprecipitated a protein of about 20 kDa (lane 4), as predicted for the extracellular ULBP1 region, which was not observed in control immunoprecipitations using mouse IgG1 (lane 2) or NKG2D receptor-specific mAbs (lane 3). Rh ULBP1/Fc is loaded as a control (lane 1).

VI. APPENDIX

1. Manuscript in preparation: Post-transcriptional regulation of ULBP1 ligand for the activating immunoreceptor NKG2D involves 3' untranslated region

Post-transcriptional regulation of ULBP1 ligand for the activating immunoreceptor NKG2D involves 3' untranslated region

Heike Himmelreich[a], Arina Mathys[a], Aleksandra Wodnar-Filipowicz[a] and Christian P. Kalberer[a]

[a] Experimental Hematology, Department of Biomedicine, University Hospital Basel, Switzerland

Corresponding author: Christian Kalberer, Experimental Hematology, Department of Biomedicine, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland; phone: ++41-61-2652333, FAX: ++41-61-2652350, e-mail: ckalberer@uhbs.ch

Short title: Post-transcriptional regulation of ULBP1

Abstract

The immunoreceptor NKG2D expressed by NK and T cells allows recognition and lysis of tumor cells expressing the corresponding ligands. To spare healthy cells from NKG2D-mediated cytotoxicity, the expression of NKG2D ligands (NKG2D-L) must be tightly regulated. Discrepancy between NKG2D-L mRNA and surface expression levels indicates that post-transcriptional mechanisms may be involved in the regulation of NKG2D-L expression in healthy and malignant cells. To examine the contribution of 3'- untranslated region (UTR) of ULBP1 to the regulation of ULBP1 gene expression, we constructed Renilla luciferase reporter plasmids containing the full-length 3'UTR of ULBP1. We show that the luciferase activity is strongly reduced to 7% - 13% in Jurkat and HeLa cells. Fragments of the 3'UTR, which cover the entire 2.4 kb of the 3'UTR, were less effective in reducing luciferase activity than the full-length 3'UTR (19% - 40%). In luciferase reporter assays, mutation of potential AU-rich elements revealed a mRNA stabilizing effect of these sequences. Mutations of potential microRNA binding sites and partial silencing of Drosha protein by short hairpin RNAs gave inconclusive results, and specific microRNAs controlling ULBP1 expression could not be identified. Altogether these results provide evidence of a novel, 3'UTR-mediated mechanism for regulation of ULBP1 and suggest that ULBP1 expression could be targeted at post-transcriptional level to enhance immunogenicity of tumor cells.

Introduction

The activating immunoreceptor NKG2D is expressed by cytotoxic NK and $CD8^+$ T cells and triggers cellular cytotoxicity and cytokine production [1]. Ligands of NKG2D (NKG2D-L) are frequently overexpressed by virus-infected or transformed cells but are rarely detectable on healthy cells.[2] The functional importance of NKG2D and its ligands in cancer immunity is illustrated by accelerated tumor development in NKG2D-deficient mice [3] and efficient rejection of tumors ectopically expressing NKG2D-L on their surface [4, 5]. In healthy tissue, inappropriate overexpression of NKG2D-L can trigger autoimmunity [6, 7]. This indicates that NKG2D-L expression levels have to be tightly regulated to allow recognition of tumor targets, but at the same time avoid destruction of untransformed tissues.

Human NKG2D-L include the MHC class I chain-related proteins A and B (MICA and MICB) and six members of the UL-16 binding protein (ULBP) family [8-10]. Despite the existence of a multitude of ligands for one receptor, selective expression of single proteins has often been observed in various cell types and in response to various inducing signals. For example, activation of T cells and macrophages leads to upregulation of MICA, but not MICB [11], and ULBP1, but not the other ULBP ligands, are expressed in differentiating myeloid progenitors and EBV-infected B cells [12, 13]. In cancer, expression of ULBP3 is specifically detectable on chronic lymphocytic leukemia cells [14], and ULBP2 is found in melanoma metastases [15] or ovarian carcinoma cells [16]. These selective expression patterns argue for the existence of diverse regulatory mechanisms specifically controlling expression of individual NKG2D-L, dependent on pathophysiological conditions, thus ensuring the exposure of potentially harmful cells to the immune effectors [17, 18].

The therapeutic induction of human NKG2D-L in cancer or infection is of considerable interest, but clinical approaches remain restrained by limited understanding of the precise mechanisms that control expression of individual ligands. The promoters of MICA and MICB genes contain regulatory elements responsible for heat shock- and oxidative stress-responses associated with the transcriptional upregulation of MIC levels upon cellular stress [19]. Recently, the post-transcriptional control of MIC expression has been described, involving the 3' untranslated region (3'UTR) of MICA and MICB genes recognized by microRNAs. Two short sequence segments conserved within the 3'UTR of MICA and MICB contain binding sites for endogenous cellular and also viral-encoded microRNAs (hsa-miR-17-5p/-20a/-93/-106b/-373/-520d and hcmv-miR-UL122) [20, 21].

In case of ULBP expression, transcriptional regulation was postulated by the analysis of the putative promoter regions [22], and binding sites for the transcription factors Sp1, Sp3 and AP-2a were specifically identified within the promoter of ULBP1 [23]. Expression of ULBP mRNA in the absence of proteins detectable on the surface of various cell types [24-27] is indicating that, in analogy to MIC ligands, not only transcriptional but also post-transcriptional mechanisms may regulate the levels of ULBPs [28].

Here we investigated the role of 3'UTR in regulation of ULBP1 expression. Considerable differences in length and sequence of the 3'UTRs of the ULBP genes suggest that this region plays a role in differential expression of ULBPs. Sequence analysis of 2.4 kb-long 3'UTR of ULBP1 revealed potential binding sites for microRNAs and AU-rich elements (ARE), the regulatory components of RNA degradation and translational suppression [29, 30]. Using luciferase reporter assays, we show that the full-length 3'UTR of ULBP1 is markedly involved in regulation of expression of ULBP1 in two cancer cell lines, HeLa and Jurkat. This finding reinforces the role of post-transcriptional mechanisms in modulating the NKG2D-L levels, which may increase the visibility of tumor cells to the effectors of the immune system.

Material and Methods

Cells

Human cervix carcinoma HeLa cells and T cell leukemia Jurkat cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and Penicillin/Streptomycin (Invitrogen).

Cloning of Luc-expression vectors

The full length 3'UTR of ULBP1 (ULBP1-3'UTR) was amplified by PCR from full-length cDNA clone IRATp970D01103D (imaGenes, Berlin, Germany) with primers containing NotI restriction sites, subcloned into pGEM-T vector (Promega, Madison, WI) and inserted into the NotI site of the Renilla luciferase reporter vector pRL-con [31] (kindly provided by W. Filipowicz, FMI, Basel) immediately downstream of luciferase coding region to produce pRL-U1-UTR. Nine fragments U1-1 to U1-9 were generated by specific PCR amplification from ULBP1-3'UTR cDNA clone with fragment-specific forward and reverse primers (Supplementary Table 1) and inserted into pRL-con. Mutated fragments of ULBP1-3'UTR, containing base pair substitutions or deletions in potential microRNA seed sequences or in

predicted ARE sites, as indicated in the Result section, were ordered from DNA 2.0 (Menlo Park, CA) and inserted into NotI sites of pRL-con. All products were verified by sequencing.

For stable luciferase expression, we used the lentiviral vector MA1 containing two expression cassettes driven by a bidirectional promoter [32] (kindly provided by L. Naldini, Milano). The ΔLNFGR gene was excised by digest with XmaI and SalI and replaced by the Renilla expression cassette. The Renilla luciferase gene with and without full-length ULBP1-3'UTR was amplified from pRL-U1-UTR with primers containing XmaI and SalI restriction sites, subcloned into pGEM-T and inserted into the lentiviral vector MA1 to produce LV-RL-U1-UTR and LV-RL-con, respectively. Lentiviruses were produced as described [33].

Luciferase assay

Jurkat cells ($6x10^5$ cells/well) were transfected in duplicates in 24-well plates with 20ng of luciferase reporter vectors and 250ng of the firefly luciferase vector pGL4.13 (Promega), using Lipofectamine 2000 (Invitrogen). HeLa cells ($4x10^4$ cells/well) were plated in 24-well plates one day prior to transfection with 20ng of reporter and 150ng of firefly luciferase vector. pRL-con served as control reporter vector. Renilla and Firefly activities were measured 48 hours after transfection using the Dual-Luciferase Reporter Assay (Promega) in a MicroLumat Plus reader (Berthold Technologies, Bad Wildbad, Germany). Renilla luciferase was normalized to firefly luciferase activity and then to the average activity of pRL-con. In cells stably transduced with LV-RL-U1-UTR, Renilla luciferase activity was measured with the Renilla Luciferase Assay (Promega) using equal cell numbers and normalized to the percentage of GFP expressing cells. Paired Student's t-test was used to compare the effect of different reporter vectors on luciferase activity.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction was performed in duplicates using *Power* SYBR Green PCR master mix in an ABI Prism 7000 (Applied Biosystems) and conditions as described [34]. The following primer pairs were used for quantification of specific mRNAs: ULBP1 forward: 5'-GTACTGGGAACAAATGCTGGAT-3', reverse: 5'-AACTCTCCTCATCTGCCAGCT-3' [35]; MICA forward: 5'-ACAATGCCCCAGTCCTCCAGA-3', reverse: 5'-ATTTTAGATATCGCCGTAGTTCCT-3 [36]; Drosha forward: 5'-GCAGTTATTTGGACGCTTGC-3', reverse: 5'-

AGTTGTCGATCAGTATTTGGC-3'; RPL19 forward: 5'-GATGCCGGAAAAACACCTTG-3', reverse: 5'-TGGCTGTACCCTTCCGCTT-3' [34]. PCR reactions were normalized to RPL19 using the $2^{-\Delta\Delta Ct}$ method [34].

Stem-loop quantitative RT-PCR to quantify expression levels of mature miRNAs (TaqMan microRNA Assays, Applied Biosystems) was performed according to the manufacturer's instructions in an ABI Prism 7500 Real-Time PCR system (Applied Biosystems). PCR reactions were run in duplicates, and miRNA expression, relative to hsa-miR-16, was calculated [34]. The threshold of microRNA expression level was defined as ΔCT(miR-16 – miR-X) >12.

MicroRNA overexpression

Complementary oligonucleotides designed to form artificial pre-miRNA hairpins (Supplementary Table 2; ordered from Eurogentec, Seraing, Belgium), were annealed by heating to 95°C for 5 minutes and slow cooling to room temperature, phosphorylated with T4 polynucleotide kinase and inserted into the HindIII and BglII sites of a modified pSUPER vector [37]. The vectors were transiently transfected into Jurkat cells with indicated concentrations, using Lipofectamine 2000, and FACS, luciferase assay and RNA isolation was performed 48 hours later.

Drosha knockdown with short hairpin RNA

Lentiviral vectors containing a Drosha-specific shRNA (LV-shDrosha) or a control shRNA (LV-shControl), as well as a GFP expression cassette were kindly provided by O. Mandelboim, Jersualem, Israel) [20, 21]. Transduction efficiency of HeLa cells was assessed by measuring GFP^+ cells by FACS. NKG2D-L surface expression and RNA levels were measured five days after transduction. To perform the luciferase assay, HeLa cells were transduced with LV-RL-U1-UTR or LV-RL-con, sorted for GFP expression and subsequently transduced with LV-shDrosha or LV-shControl. Seven days upon the second transduction, luciferase activity was measured as described above.

Flow cytometry

Cells were stained with unconjugated mouse monoclonal antibodies (mAbs) against ULBP1 (M295; kind gift of D. Cosman, Amgen, Seattle, WA), MICA/B, HLA class I or with mouse IgG1 isotype (BD Biosciences, Allschwil, Switzerland) at 10 µg/mL and with secondary goat-anti mouse IgG-FITC (Jackson ImmunoResearch, West Grove, PA) or goat-anti mouse IgG-

Alexa647 (Molecular Probes, Invitrogen). Propidium iodide (Sigma, Buchs, Switzerland) was used to exclude dead cells from analysis. At least 20'000 events were acquired using FACS (FACSCalibur; BD Biosciences) and analysis was performed using FlowJo software (TreeStar, Ashland, OR). Surface expression level of NKG2D-L was defined as the mean fluorescence intensity ratio (MFIR) of values obtained with specific mAbs divided by values given by secondary or control mAbs [12].

Results

Regulation of ULBP1 expression by 3'UTR

Since various cells and tissues were shown to express ULBP1 mRNA in the absence of ULBP1 protein detectable on the cell surface, these discrepancies are suggestive of the involvement of post-transcriptional mechanisms in regulating ULBP1 levels. Here we studied the role of the 2.4-kb-long 3'UTR in post-transcriptional regulation of ULPB1 gene expression. The reporter plasmid pRL-U1-UTR, which contains the luciferase gene fused to the full-length ULBP1-3'UTR, was transiently transfected into HeLa and Jurkat cells. The luciferase activity was significantly decreased to 7%-13% compared to the control plasmid pRL-con without ULBP1-3'UTR (Figure 1A) Likewise, following stable transduction of HeLa and Jurkat cells with lentiviral vectors containing the luciferase ULBP1-3'UTR expression cassette (LV-RL-U1-UTR), we observed a strongly reduced luciferase signal compared to a control vector LV-RL-con (Figure 1B). These results indicate that the 3'UTR contains regulatory sequences for ULBP1 gene expression.

To determine the position of regulatory sequences in the ULBP1-3'UTR, we generated nine constructs pRL-U1-1 to pRL-U1-9 carrying fragments of ULBP1-3'UTR inserted downstream of the luciferase gene (Figure 2A). Upon transient expression in HeLa and Jurkat cells, all fragments led to significant reductions of luciferase activity, as compared to pRL-con (Figure 2B). The strongest effects were observed with the 5'-terminal 1052 bp-long pRL-U1-3 (19%±2% and 24%±5% in HeLa and Jurkat cells, respectively), and with the 3'-terminal 669 bp-long pRL-U1-9 (35%±4% and 26%±1%). Within pRL-U1-3, the reduction was well pronounced with the 367 bp-long fragment U1-5 (40%±3% and 37%±4%), and the short, 178bp, U1-6 sub-fragment thereof (40%±4% and 40%±1%). Interestingly, none of the fragments was as potent as the full-length ULBP1-3'UTR, and the effects were seen with every fragment along the 3'UTR, suggesting that the regulatory sequences are distributed over

the entire 3'UTR, rather than restricted to specific areas. By examining the sequence of ULBP1-3'UTR, we identified a number of potential regulatory elements, including microRNA binding sites and ARE motifs, and we next investigated their involvement in regulating the levels of ULBP1.

Role of ARE in regulation of ULBP1 expression

Sequence analysis of the ULBP1-3'UTR indicated the presence of four potential ARE, three of them located in fragment U1-5 and one in fragment U1-9. ARE motifs within the 3' UTR of mRNAs are crucial determinants of transcript instability in mammalian cells [30]. To determine the contribution of these elements to ULBP1 post-transcriptional regulation, we generated mutated fragments of U1-5 and U1-9 (Figure 3A) in which the ARE were altered by base pair substitutions, giving rise to vectors pRL-U1-5-AREmut1 (ATTTA to ATGTA) and pRL-U1-5-AREmut2 (ATTTA to AGGGA) or pRL-U1-9-AREmut2 (ATTTA to AGGGA). In addition, we mutated an ARE-like site ATTTTA located in fragment U1-5 (to ATGTTA or AGGGGA; see also Supplementary Figure S1). Following transfection of unmutated pRL-U1-5 and pRL-U1-9 and their mutated counterparts to HeLa and Jurkat cells, we found that the mutations did not lead to an increase in luciferase activity (Figure 3B), as would be expected from inactivation of RNA destabilizing sites. Instead, the luciferase activities were reduced with individual mutated constructs in both HeLa and Jurkat cells (to 60.5% - 86.6% of control vectors; Figure 3B). These results indicate that ARE sites are not responsible for reduced luciferase activity with U1-5 and U1-9 constructs, and it is therefore unlikely that they are involved in the suppression of ULBP1 transcript levels.

Role of microRNAs in regulation of ULBP1 expression

We next examined the role of microRNA-binding sites as regulatory elements within the ULBP1-3'UTR. MicroRNAs are important players in post-transcriptional regulation [38, 39]. In an attempt to identify the microRNAs that target ULBP1 mRNA, we used computer algorithms to search the ULBP1-3'UTR for potential sites recognized by cellular microRNAs. The accuracy of programs to predict microRNA binding sites in ULBP1-3'UTR is limited because human ULBP1 does not show sequence conservation with NKG2D ligands from other species, which is one of the criteria used by most of these programs. Using the four different programs, TargetScanHuman v.4.2 and v5.1[40], DIANA microT v3.0 [41], ElMMO v3 [42], and Micro-Inspector v1.5 [43], more than 200 potential microRNA were predicted to target the ULBP1-3'UTR. By focusing on U1-6 and U1-9 fragments, as based on their strong

suppressing effect on luciferase activity (see Figure 2B), we selected 13 microRNAs predicted by more than one program to bind to U1-6 or U1-9, or to have at least two binding sites in the full-length ULBP1-3'UTR (Supplementary Figure S2).

First we examined whether any of these candidate microRNAs are expressed in Jurkat and HeLa cells. Using quantitative PCR we found miR-140 and miR-650 expression in Jurkat cells and miR-140, miR-409, miR-433 and miR-650 in HeLa cells (Figure 4A). All other miRNAs were either not present or were weakly expressed below the threshold defined according to miR-16, used in the assay as the housekeeping microRNA. To determine whether candidate microRNAs are involved in recognition of ULBP1-3'UTR and lead to reduced reporter luciferase activity, we generated mutants of fragment U1-6 by introducing base pair deletions or substitutions which should prevent the pairing of miR-140/-380/-409/-433/-650 to the predicted binding sites (pRL-U1-6sub and pRL-U1-6del constructs: see Supplementary Figure S3). In comparison to unmutated pRL-U1-6, mutated vector pRL-U1-6sub produced a statistically significant increase in luciferase activity in Jurkat cells, and also all other mutated fragments showed a similar tendency (Figure 4B), suggesting that at least some of the candidate microRNAs expressed in HeLa and Jurkat cells may contribute to regulation of ULBP1 within the region U1-6 of 3' UTR.

To validate the microRNA involvement in regulating ULPB1 transcript levels, we overexpressed several candidate microRNAs and examined whether high levels of these microRNAs lead to a decrease in ULBP1 surface expression. By transient co-transfection of a mix of six microRNA-overexpressing vectors (pSUPER-miR-140/-380/-381/-409/-433 and -650) into Jurkat cells, we achieved microRNA levels that were 2.5-fold (miR-381) to 252-fold (miR-650) higher than the endogenous levels of respective microRNA (Figure 5A). When microRNA overexpressing plasmids were co-transfected with luciferase reporter vectors (pRL-U1-UTR or the pRL-U1-6 or U1-9), the luciferase activity was not altered (Figure 5B). No reduction of luciferase activity in the presence of increased microRNA levels is arguing against the involvement of these selected microRNAs in regulating ULPB1 transcript levels. Consistent with this observation, also the surface expression of ULBP1 protein was not affected in pSUPER-miR transfected Jurkat cells (Figure 5C). Unchanged was also the surface expression of HLA-ABC molecules (data not shown), as an indication of lack of potential unspecific off-target effects of microRNA overexpression. Based on these results, we were unable to demonstrate a specific modulatory role of the six candidate microRNAs in the regulation of the regions U1-6 and U1-9 of ULBP1-3'UTR.

Role of Drosha in regulation of ULBP1 expression

Since the results described above did not identify specific microRNAs which are potentially involved in regulation of ULBP1 expression, we used an approach of knock-down of Drosha, a crucial component of microRNA biogenesis [44]. We transduced HeLa cells with a lentiviral vector expressing the short hairpin RNA directed against Drosha (LV-shDrosha) [21], and obtained a 2.3-fold reduction of Drosha mRNA levels (Figure 6A). In agreement with the known involvement of microRNAs in regulating MICA [21], the MICA mRNA level in transduced HeLa cells was increased by 1.4-fold (Figure 6A). However, we found no analogous increase in ULBP1 mRNA, the levels of which were even reduced by 50% (Figure 6A). Accordingly, when HeLa cells were transduced with LV-RL-U1-UTR, Drosha knock down had no effect on luciferase activity, which remained at a similarly low level of approximately 10%, as in cells transduced with LV-shControl (Figure 6B). Furthermore, ULBP1 surface expression remained low in LV-shDrosha transduced cells, while MICA surface expression was markedly up-regulated from MFIR 11.7 to 17.1 (Figure 6C). In order to assess whether HeLa cells are capable of expressing cell surface ULBP1 at enhanced levels, we performed a control experiment by transfecting cells with the ULBP1 expression vector RSV.5ULBP1 [45]. As a significant proportion of transfected cells (41%) expressed high surface levels of ULBP1 (Figure 6D), we conclude that absence of ULBP1 up-regulation in Drosha knock-down HeLa cells is not due to intrinsic limiting factors. The fact that the knock-down of Drosha was sufficient to increase MICA but not ULBP1 expression may indicate that ULBP1 is regulated by mechanisms other than microRNAs. At the same time, we cannot exclude that microRNAs which are less sensitive to Drosha downregulation could target the 3'UTR of ULBP1.

Discussion

NK and T cells constitutively express the activating receptor NKG2D at high levels. Therefore, the control of expression of the corresponding NKG2D-Ls is essential for preventing a spontaneous NKG2D-mediated cytotoxicity against healthy neighboring tissue, while directing the immune response towards infected or transformed cells. The complexity of multiple known NKG2D-Ls is also reflected in multiple mechanisms underlying the induction and expression of individual ligands at the transcriptional, post-transcriptional, and post-translational levels, as extensively demonstrated with the members of MIC ligand family [21].

In comparison with MIC ligands, knowledge on the mechanisms of expression of ULBP ligand family members remains fragmentary. In this work, we studied the involvement of post-transcriptional mechanisms in regulation of ULBP1, and we provide the first evidence that 3' UTR plays a role in regulating ULBP1 expression in cancer cells.

Among the ULBP genes, ULBP1 contains the longest, 2.4 kb-long 3'UTR [25]. The regulatory potential of this region was shown in the functional assays employing the full-length 3'UTR of ULBP1 fused to a luciferase reporter gene. Up to 14-fold suppression of luciferase activity was observed in Jurkat and HeLa cells after transient transfection as well as stable transduction with lentiviral vectors. Also fragments of ULBP1-3'UTR, which ranged from 178 – 1152 bp, had a suppressive effect, although to a lesser degree compared to the full-length 3'UTR. There was no correlation of fragment length and luciferase activity, suggesting that the regulatory elements are scattered all along the 2.4 kb-long UBLP1-3'UTR.

The apparent involvement of 3'UTR and wide distribution of the putative regulatory elements suggests microRNAs as candidate regulators of ULPB1. Using several computational algorithms [40-43], numerous potential microRNA binding sites in ULBP1 3'UTR could be predicted. Among the generated fragments of 3'UTR, we focused on fragment U1-6, because it was only 178bp long with 9 predicted microRNA binding sites, but nevertheless led to a significant 2.5-fold suppression of luciferase activity in Jurkat cells. By performing the expression profile analysis of microRNAs in HeLa and Jurkat cells, the number of candidate microRNAs was reduced to 6, and we asked whether these play a role in targeting U1-6, either by mutating the respective seed sequences or by overexpression of microRNAs. Our results showed that, on one hand, inactivation of potential microRNA binding sites in fragment U1-6 led to a significant increase of reporter activity, speaking for the involvement of these specific microRNAs in the control of luciferase expression. On the other hand, overexpression of specific microRNAs did not affect reporter activity or ULBP1 surface expression in ULBP1^{+} Jurkat cells, arguing against a role of these microRNAs in ULBP1 regulation. Also, a functionally relevant 2.3-fold reduction of Drosha mRNA by specific shRNAs, sufficient to increase MICA mRNA and cell surface levels, did not influence luciferase or ULBP1 expression. Since most of microRNA prediction algorithms use sequence conservation among species as one of their selection criteria and since ULBP1 is only found in men and anthropoid apes, the accuracy of prediction algorithms may have been insufficient to choose the appropriate microRNAs, and this may explain our inconclusive results.

Computer analysis predicted four ARE sites in the ULBP1-3'UTR, three in fragment U1-6 and one in fragment U1-9. We examined their involvement in ULBP1 regulation by mutating the ATTTA consensus sequence. The inactivation of all four ARE sequences did not lead to any increase but rather to a significant decrease of the luciferase activity. Thus our results do not corroborate the possibility that low ULBP1 surface protein levels in many cell lines and primary cells are caused by ARE-mediated mRNA degradation. Instead the data indicate that AREs of ULBP1 3'UTR may be recognized by mRNA stabilizing proteins such as the HuR protein which is expressed in all proliferating cells [46]. Interestingly a correlation of ULBP1 expression and proliferation was described [11], but no link to ARE or HuR protein levels has been made.

The search for additional regulatory elements in ULBP1-3'UTR revealed a potential Alu repeat, a non-coding RNA sequence with internal tandem duplications, which belongs to the short interspersed nucleotide elements [47]. This element at position 473-770 of ULBP1-3'UTR overlaps with fragments U1-2 to U1-6 but does not include the predicted AREs in fragment U1-6. The mechanism by which Alu elements modulate gene expression are only little understood [48], but possible interactions with microRNAs have been postulated [49]. Remarkably some microRNAs which may bind Alu sequences were among those described to regulate MICA and MICB, e.g. miR-17-5p/-20a/-93/-106b/-373/-520d [21]. It is therefore tempting to speculate that these microRNAs could also target the Alu sequence of ULBP1-3'UTR and thereby effectively prevent mRNA translation. The fact that most microRNA prediction algorithms have excluded repetitive sequences from consideration [40] would explain why the Alu-specific microRNAs were not among those listed to bind ULBP1 mRNA.

Our results add to the notion that induction of individual NKG2D-L shows distinct features, which may allow for a differential pattern of their expression. The transcriptional regulation was reported for both MIC and ULBP1, yet the contributing transcriptional factors are different [22, 23]. In this study we demonstrated that ULBP1 expression is regulated also at the post-transcriptional level, and that similarly to MIC ligands, the regulation involves the 3'UTR. Whereas specific cellular microRNAs target the 3'UTR of MICA and MICB [21], the role of microRNAs in regulation of ULBP1 remains speculative. Further differences apply to the regulation of cell surface ligand levels by shedding, shown for MICA and MICB along with a reduced immunogenicity of NKG2D-L expressing cells [35, 50], but not found with ULBP1 [51]. Given that NKG2D-L expression is crucial for tumor recognition, understanding

the different gene expression mechanisms is of importance for exploring NKG2D receptor-ligand interactions in therapeutic strategies to reinforce NK and T cell anti-tumor immunity.

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References

1. Raulet DH: Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 3(10):781, 2003.
2. Gonzalez S, Lopez-Soto A, Suarez-Alvarez B, Lopez-Vazquez A, Lopez-Larrea C: NKG2D ligands: key targets of the immune response. Trends Immunol 29(8):397, 2008.
3. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, Knoblaugh S, Cado D, Greenberg NM, Raulet DH: NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 28(4):571, 2008.
4. Cerwenka A, Baron JL, Lanier LL: Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A 98(20):11521, 2001.
5. Jamieson AM, Diefenbach A, McMahon CW, Xiong N, Carlyle JR, Raulet DH: The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity 17(1):19, 2002.
6. Groh V, Bruhl A, El-Gabalawy H, Nelson JL, Spies T: Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. Proc Natl Acad Sci U S A 100(16):9452, 2003.

7. Hue S, Mention JJ, Monteiro RC, Zhang S, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N, Caillat-Zucman S: A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 21(3):367, 2004.
8. Bahram S, Inoko H, Shiina T, Radosavljevic M: MIC and other NKG2D ligands: from none to too many. Curr Opin Immunol 17(5):505, 2005.
9. Sutherland CL, Chalupny NJ, Schooley K, VandenBos T, Kubin M, Cosman D: UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. J Immunol 168(2):671, 2002.
10. Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J: Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. J Immunol 173(2):1078, 2004.
11. Cerboni C, Zingoni A, Cippitelli M, Piccoli M, Frati L, Santoni A: Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis. Blood 110(2):606, 2007.
12. Nowbakht P, Ionescu MC, Rohner A, Kalberer CP, Rossy E, Mori L, Cosman D, De Libero G, Wodnar-Filipowicz A: Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood 105(9):3615, 2005.
13. Pappworth IY, Wang EC, Rowe M: The switch from latent to productive infection in epstein-barr virus-infected B cells is associated with sensitization to NK cell killing. J Virol 81(2):474, 2007.
14. Poggi A, Venturino C, Catellani S, Clavio M, Miglino M, Gobbi M, Steinle A, Ghia P, Stella S, Caligaris-Cappio F, Zocchi MR: Vdelta1 T lymphocytes from B-CLL patients recognize ULBP3 expressed on leukemic B cells and up-regulated by trans-retinoic acid. Cancer Res 64(24):9172, 2004.
15. Paschen A, Sucker A, Hill B, Moll I, Zapatka M, Nguyen XD, Sim GC, Gutmann I, Hassel J, Becker JC, Steinle A, Schadendorf D, Ugurel S: Differential clinical significance of individual NKG2D ligands in melanoma: soluble ULBP2 as an indicator of poor prognosis superior to S100B. Clin Cancer Res 15(16):5208, 2009.
16. Carlsten M, Bjorkstrom NK, Norell H, Bryceson Y, van Hall T, Baumann BC, Hanson M, Schedvins K, Kiessling R, Ljunggren HG, Malmberg KJ: DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. Cancer Res 67(3):1317, 2007.

17. Eagle RA, Trowsdale J: Promiscuity and the single receptor: NKG2D. Nat Rev Immunol 7(9):737, 2007.
18. Nausch N, Cerwenka A: NKG2D ligands in tumor immunity. Oncogene 27(45):5944, 2008.
19. Venkataraman GM, Suciu D, Groh V, Boss JM, Spies T: Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D. J Immunol 178(2):961, 2007.
20. Stern-Ginossar N, Elefant N, Zimmermann A, Wolf DG, Saleh N, Biton M, Horwitz E, Prokocimer Z, Prichard M, Hahn G, Goldman-Wohl D, Greenfield C, Yagel S, Hengel H, Altuvia Y, Margalit H, Mandelboim O: Host immune system gene targeting by a viral miRNA. Science 317(5836):376, 2007.
21. Stern-Ginossar N, Gur C, Biton M, Horwitz E, Elboim M, Stanietsky N, Mandelboim M, Mandelboim O: Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. Nat Immunol 9(9):1065, 2008.
22. Eagle RA, Traherne JA, Ashiru O, Wills MR, Trowsdale J: Regulation of NKG2D ligand gene expression. Hum Immunol 67(3):159, 2006.
23. Lopez-Soto A, Quinones-Lombrana A, Lopez-Arbesu R, Lopez-Larrea C, Gonzalez S: Transcriptional regulation of ULBP1, a human ligand of the NKG2D receptor. J Biol Chem 281(41):30419, 2006.
24. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T: Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A 93(22):12445, 1996.
25. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ: ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14(2):123, 2001.
26. Boissel N, Rea D, Tieng V, Dulphy N, Brun M, Cayuela JM, Rousselot P, Tamouza R, Le Bouteiller P, Mahon FX, Steinle A, Charron D, Dombret H, Toubert A: BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. J Immunol 176(8):5108, 2006.
27. Nedvetzki S, Sowinski S, Eagle RA, Harris J, Vely F, Pende D, Trowsdale J, Vivier E, Gordon S, Davis DM: Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. Blood 109(9):3776, 2007.

28. Cerwenka A: New twist on the regulation of NKG2D ligand expression. J Exp Med 206(2):265, 2009.
29. Filipowicz W, Bhattacharyya SN, Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9(2):102, 2008.
30. Barreau C, Paillard L, Osborne HB: AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res 33(22):7138, 2005.
31. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W: Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309(5740):1573, 2005.
32. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L: Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nat Biotechnol 23(1):108, 2005.
33. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D: Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15(9):871, 1997.
34. Kralovics R, Buser AS, Teo SS, Coers J, Tichelli A, van der Maas AP, Skoda RC: Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. Blood 102(5):1869, 2003.
35. Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, Rammensee HG, Steinle A: Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 102(4):1389, 2003.
36. Borchers MT, Harris NL, Wesselkamper SC, Vitucci M, Cosman D: NKG2D ligands are expressed on stressed human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 291(2):L222, 2006.
37. Schomber T, Kalberer CP, Wodnar-Filipowicz A, Skoda RC: Gene silencing by lentivirus-mediated delivery of siRNA in human CD34+ cells. Blood 103(12):4511, 2004.
38. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281, 2004.
39. Chen K, Rajewsky N: The evolution of gene regulation by transcription factors and microRNAs. Nat Rev Genet 8(2):93, 2007.
40. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: Prediction of mammalian microRNA targets. Cell 115(7):787, 2003.

41. Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, Hatzigeorgiou A: A combined computational-experimental approach predicts human microRNA targets. Genes Dev 18(10):1165, 2004.
42. Gaidatzis D, van Nimwegen E, Hausser J, Zavolan M: Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinformatics 8:69, 2007.
43. Rusinov V, Baev V, Minkov IN, Tabler M: MicroInspector: a web tool for detection of miRNA binding sites in an RNA sequence. Nucleic Acids Res 33(Web Server issue):W696, 2005.
44. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN: The nuclear RNase III Drosha initiates microRNA processing. Nature 425(6956):415, 2003.
45. Steinle A, Li P, Morris DL, Groh V, Lanier LL, Strong RK, Spies T: Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. Immunogenetics 53(4):279, 2001.
46. Peng SS, Chen CY, Xu N, Shyu AB: RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. Embo J 17(12):3461, 1998.
47. Batzer MA, Deininger PL: Alu repeats and human genomic diversity. Nat Rev Genet 3(5):370, 2002.
48. Hasler J, Strub K: Alu elements as regulators of gene expression. Nucleic Acids Res 34(19):5491, 2006.
49. Smalheiser NR, Torvik VI: Alu elements within human mRNAs are probable microRNA targets. Trends Genet 22(10):532, 2006.
50. Kaiser BK, Yim D, Chow IT, Gonzalez S, Dai Z, Mann HH, Strong RK, Groh V, Spies T: Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands. Nature 447(7143):482, 2007.
51. Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, Hofsteenge J, Gratwohl A, Tichelli A, Paluszewska M, Wiktor-Jedrzejczak W, Kalberer CP, Wodnar-Filipowicz A: NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. Blood 111(3):1428, 2008.

Figure legends

Figure 1. Scheme of luciferase reporter constructs and luciferase activity in HeLa and Jurkat cells. (A) The 2.4 kb full length 3'UTR of ULBP1 was inserted downstream of a luciferase open reading frame into the parental vector pRL-con, resulting in pRL-U1-UTR. Luciferase activities of pRL-con (light grey) and pRL-U1-UTR (dark grey) are shown as means ± SEM (n=14 for HeLa, n=20 for Jurkat). (B) The 2.4 kb full-length 3'UTR of ULBP1, fused to the luciferase gene, or the luciferase gene alone, were inserted into a lentiviral vector (LV-RL-con and LV-RL-U1-UTR, respectively). Luciferase activity was measured 7 days after transduction with LV-RL-con (light grey) and LV-Rl-U1-UTR (dark grey). Results are means ± SEM (n=8 for HeLa and n=5 for Jurkat).

Figure 2. Luciferase activity of reporter plasmids containing fragments of U1-3'UTR upon transient transfection into HeLa and Jurkat cells. Results were normalized to pRL-con and shown as means ± SEM (n=2-14 for HeLa, n=2-20 for Jurkat). prom = promoter; luc = luciferase.

Figure 3. Mutation of ARE in two regions of the U1-3'UTR. (A) Schematic localization of ARE in fragment U1-5 and U1-9 of the U1-3'UTR. The sequence motifs ATTTA (black square) and ATTTTA (white square) were mutated by single base substitutions T→G (triangles) or by substitution of three bases TTT→GGG (black cirlce) and four bases TTTT→GGGG (white circle), respectively. Mutated fragments were inserted into pRL-con. (B) Luciferase activity was measured upon transient transfection of wild-type and mutated pRL-U1-5 (light grey) and pRL-U1-9 (dark grey) into Hela and Jurkat cells and normalized to the activity of the corresponding wild-type reporter vectors (pRL-U1-5 and pRL-U1-9, respectively). Results are means ± SEM of duplicates (n=2-5). * = $p < 0.05$; ** = $p < 0.01$.

Figure 4. Expression of candidate microRNAs and effect of seed sequence mutations on luciferase activity. (A) Endogenous expression levels of 13 candidate microRNAs were measured in HeLa (light grey) and Jurkat cells (dark grey). Results are means ± SEM (n=1-5 in HeLa and Jurkat). (B) Luciferase activity was measured upon transient transfection of luciferase vectors, containing seed sequence mutations in fragment U1-6 (pRL-U1-6 2sub and pRL-U1-6 4del, respectively, see Supplementary Figure S2), into HeLa (light grey) and

Jurkat cells (dark grey). Luciferase activity was normalized to the activity of the non-mutated pRL-U1-6 vector. Results are means ± SEM (n=2 for HeLa and Jurkat). * = $p < 0.05$.

Figure 5. Overexpression of candidate microRNAs in Jurkat cells. (A) MicroRNA expression levels were measured upon transient transfection of 300ng pSuper empty (light grey) or co-transfection of six microRNA overexpression vectors (6x50ng pSuper mix; dark grey) into Jurkat cells. Results are means ± SEM. (B) Luciferase activity was measured upon co-transfection of 300ng pSuper empty vector (light grey) or 6x50ng pSuper mix (dark grey) with luciferase reporter vectors. Luciferase activity was normalized to the activity of pRL-con. Results are means ± SEM. (C) FACS analysis of ULBP1 surface expression in Jurkat cells transfected with pSuper empty (shaded histogram) or pSuper mix (thick line). Thin line: staining with isotype control Ab and secondary antibody.

Figure 6. Downregulation of Drosha with shRNA in HeLa cells. (A) Measurement of Drosha, ULBP1 and MICA mRNA levels upon transduction with LV-shDrosha. The expression level of each mRNA was normalized to the corresponding mRNA expression level upon transduction with LV-shCon. (B) The luciferase activity was measured upon co-transduction of LV-shCon (light grey) or LV-shDrosha (dark grey) and lentiviral luciferase reporter constructs (LV-RL-con and LV-RL-U1-UTR). Luciferase activity was normalized to the amount of GFP^+ cells and then normalized to the luciferase activity of the control vector (LV-RL-con). Results are means ± SEM. (C) FACS analysis of ULBP1 surface expression on Hela cell upon transduction with LV-shCon (shaded histogram) or LV-shDrosha (thick line). Thin line: staining with isotype control Ab and secondary antibodies. (D) FACS analysis of ULBP1 surface expression upon transfection with RSV5.ULBP1 overexpression vector.

Supplementary Figure S1. Sequence alignment of fragment U1-5 and U1-9 with wild type ARE and mutated AREs.

Supplementary Figure S2. Predicted binding sites of selected microRNA in ULBP1-3'UTR.

Supplementary Figure S3. Sequence alignment of fragment U1-6 with predicted and mutated microRNA seed sequences.

Supplementary Table S1. Forward and reverse primer sequences used to clone fragments U1-1 to U1-9 from ULBP1 3'UTR.

Supplementary Table S2. Forward and reverse primer sequences used to generate microRNA overexpression vectors.

2. Figures of the manuscript

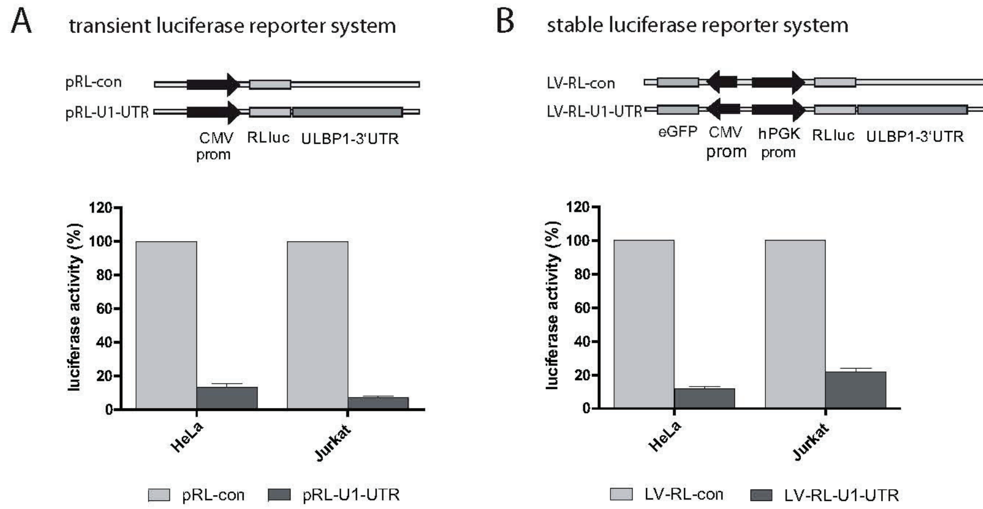


Fig.1 Himmelreich et al.

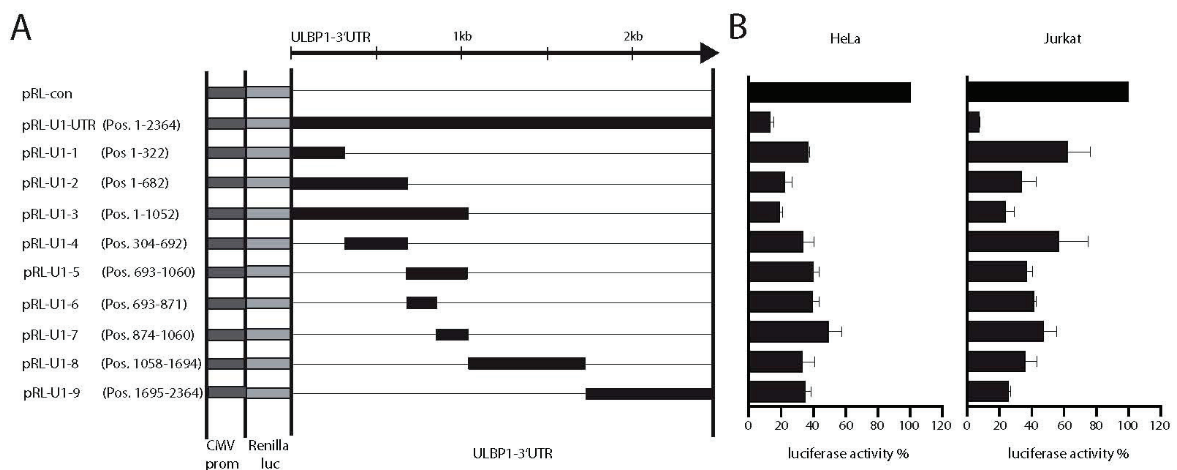


Fig. 2 Himmelreich et al.

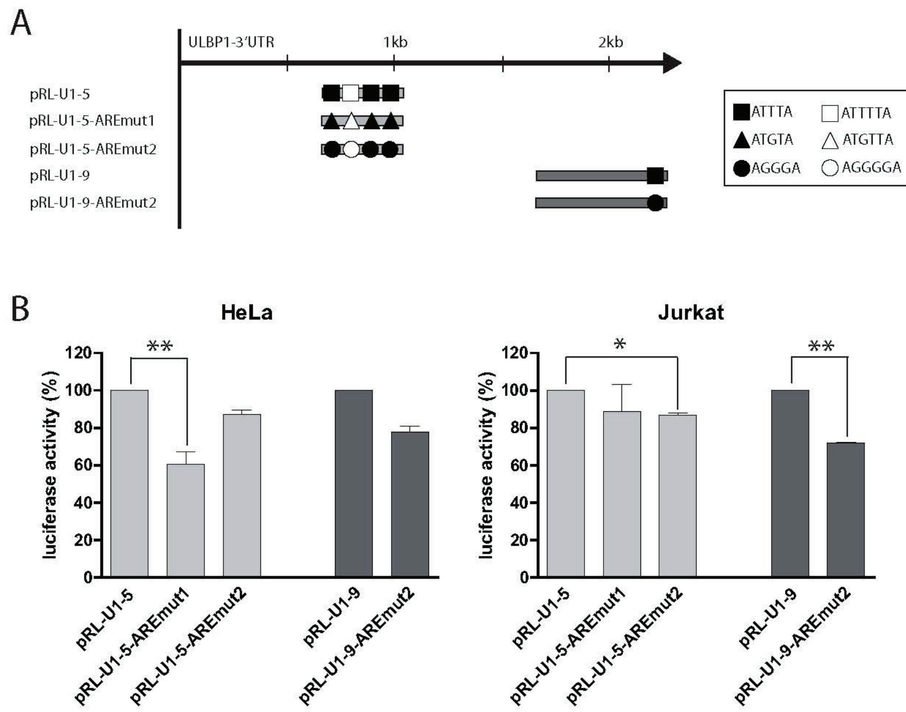
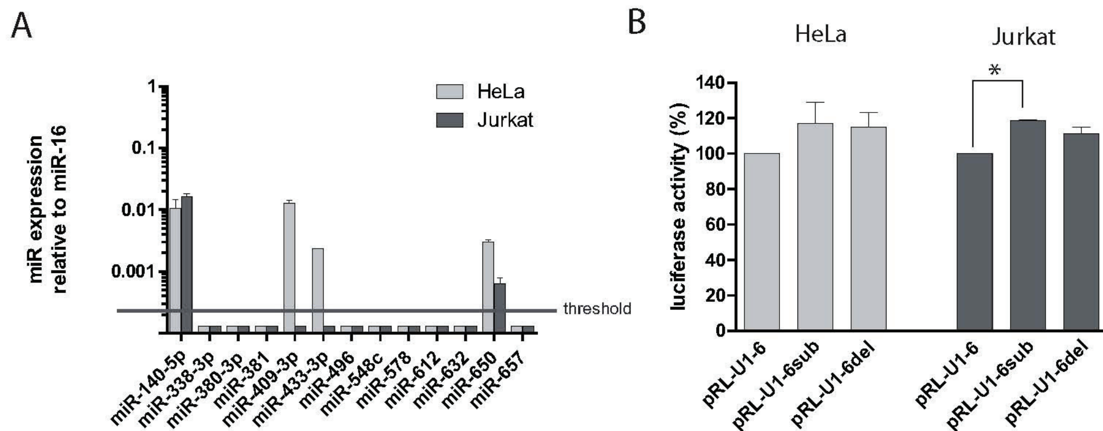


Fig. 3 Himmelreich et al.



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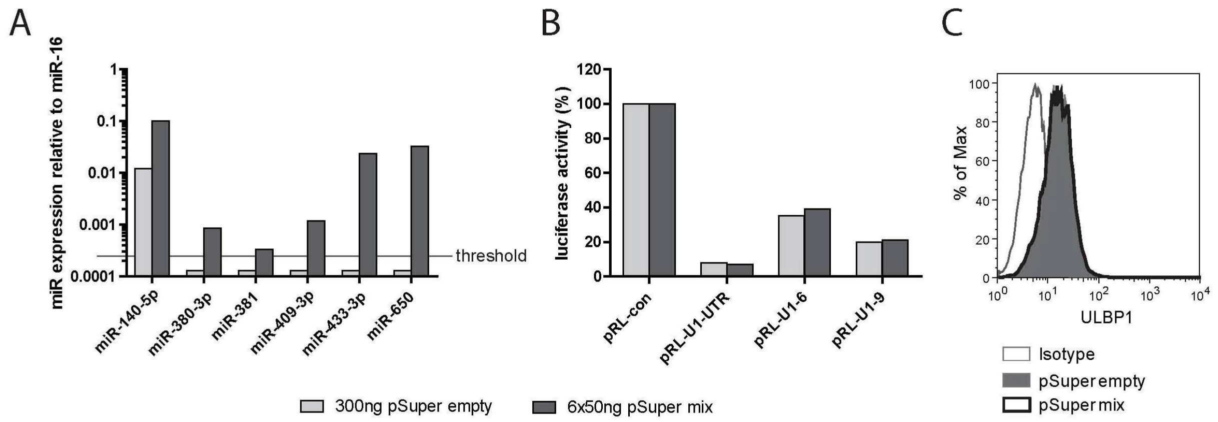

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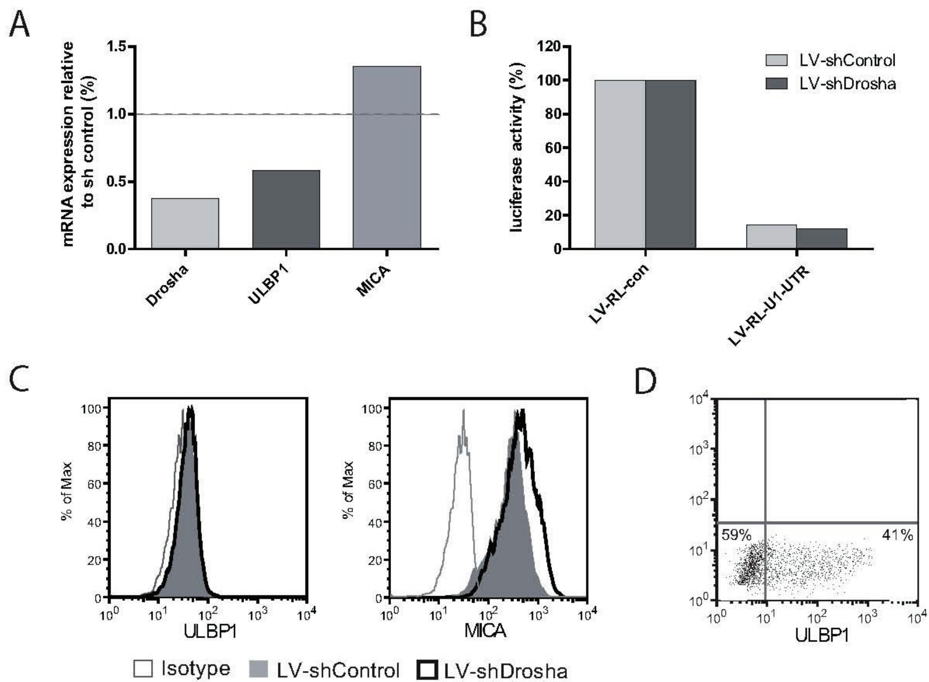

Fig. 6 Himmelreich et al.

A

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U1-5 WT   5'  AAGTCGACGC GGCCGCTTCA AATCTTGGTC TCGAGCAATG CTACCACCTT GGCCTCCCAA  3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5 WT   5'  TGCTCTGGGA TTACAGACAT GAACCACAGT GCCTGTTGTA GAAATTTTTA ATTATTTAAT  3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---------- -----G----  3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----GGG---  3'

U1-5 WT   5'  ATGAAAATAT TACATTCATG ATTATTTTAT TTAGTAAATA AAATAATAGA GAGCCCAGAA  3'
U1-5 mut  5'  ---------- ---------- -----G---- G--------- ---------- ----------  3'
U1-5 mut2 5'  ---------- ---------- ----GGGG-G GG-------- ---------- ----------  3'

U1-5 WT   5'  ATCAACCTGC ACACCTACCG CCATCTAATC TTCAATAGAA ATGGGCAATG TGGGAAAGAC  3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5 WT   5'  TCCCTATTCG AAAATTAGTG CTGGAATATC TGGCCAACCA TATGCAGAAG AATGAAACTG  3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---------- ----------  3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-5 WT   5'  AACCCCTACT TCTCCCCATA TATGTAAAAT AATTCAATAT GGATGAAAGA TTTAAATATA  3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---------- -G--------  3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---------- GGG-------  3'

U1-5 WT   5'  AGTACTAAAA CTGTAAAAAT CCTGGAGCGG CCGCAGTCGA CAA                    3'
U1-5 mut  5'  ---------- ---------- ---------- ---------- ---                    3'
U1-5 mut2 5'  ---------- ---------- ---------- ---------- ---                    3'
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B

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U1-9 WT   5'  AAGTCGACGC GGCCGCGTGC TATTCTCACC AGCAAGGACA GAGAATCAAT CTAAGTGCCC  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  AACAACAGTA AATTCAATGA AAAAAAATGT GGTACATAGA TACGATGGAA AACTATGCAG  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  CCATGAAACA CAAGAAAATC ATGTCCTTTT CAGCAACATG GATGCAACTA GAGGCTATTA  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  TCCTAAGCAA CCTAATGCAA GAACAGAAAA CCACATACTG CATCTTCCCA TTGGAAAGTG  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  GCAGCTAAAC ATTAAATTCG CATGAACCAC AGATGCTGGA GATCACCAGA CCGGGGAGAG  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  AAGAGGGGCA CCTGGGCTGA AAAACACACC TGTTGGGTAT CATGCTTACT GTCTGGGCGA  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  TGGGATCATT GGGACACCAA GCCTCAGCTT CTCAAATTCT ACCCATGTAA CAAACCTGTA  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  TATGTACCTT GTATTATATA GGTTGAAATT AAAGATGAAT AAATAAAATA AAATGACACA  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  AGGCCAAAAA CAAATGGGTT TAACTGACCA GAGCGAGAG AACTCTGCACT ATGAACCCAA  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  ACCCAGCTCA AAAAGATAAA ATCTAGTCAT TTAAGATAAT CATAAGTTGT ATGATGATAA  3'
U1-9 mut2 5'  ---------- ---------- ---------G GG-------- ---------- ----------  3'

U1-9 WT   5'  TTGTATAAAA ATTTGTATGA TGATAATTGT ATAATAATTA TACATGAAAG TGCCAAAACC  3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---------- ----------  3'

U1-9 WT   5'  CTACAATTAA ACACTGTATA ATGGAAGCGG CCGCAGTCGA CAA                    3'
U1-9 mut2 5'  ---------- ---------- ---------- ---------- ---                    3'
```

Fig. S1 Himmelreich et al.

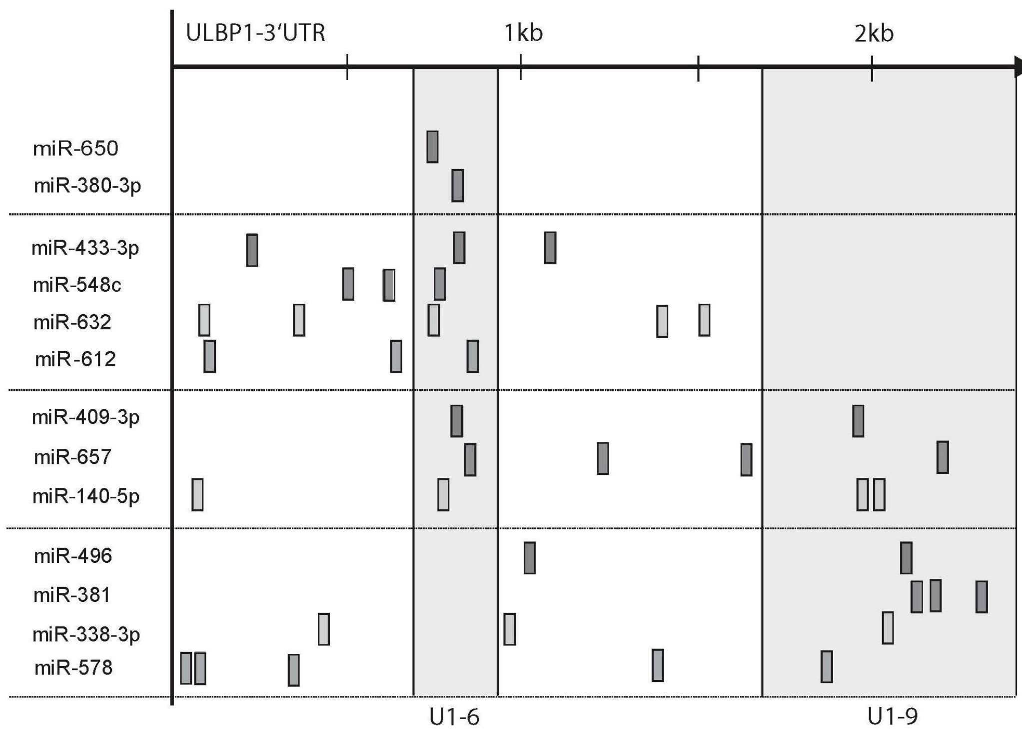

Fig. S2 Himmelreich et al.

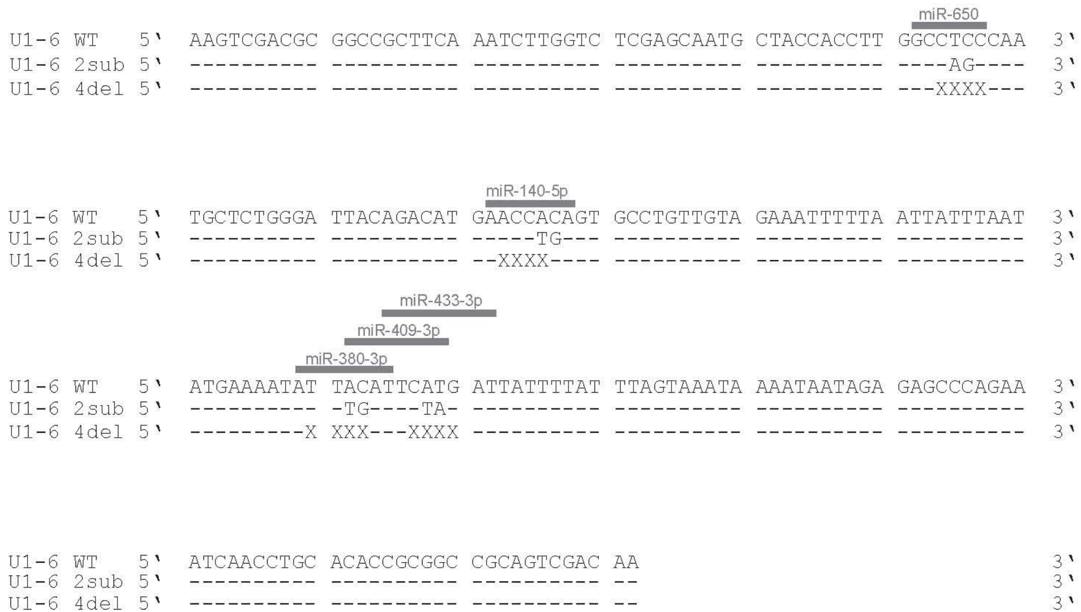

Fig. S3 Himmelreich et al.

vector system	fragment	orientation	sequence
transient luciferase reporter system	full length, U1-1, U1-2, U1-3	for	5' AA GCGGCCGC GGA GAG TTG TTT AGA GTG ACA G 3'
	full length, U1-9	rev	5' AA GCGGCCGC TTC CAT TAT ACA GTG TTT AAT TG 3'
	U1-1	rev	5' AA GCGGCCGC GCC TTT TGC TGG GAT TTC TTG TT 3'
	U1-2	rev	5' AA GCGGCCGC GGC AAC ATA GGA AGA CCC TAC CT 3'
	U1-3	rev	5' AA GCGGCCGC TTA CAG TTT TAG TAC TTA TAT TT 3'
	U1-4	for	5' AA GCGGCCGC AAG AAA TCC CAG CAA AAG GCA TT 3'
	U1-4	rev	5' AA GCGGCCGC GAC CAT CCT GGG CAA CAT AGG AA 3'
	U1-5	for	5' AA GCGGCCGC TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-5	rev	5' AA GCGGCCGC TCC AGG ATT TTT ACA GTT TTA GT 3'
	U1-6	for	5' AA GTCGAC GCGGCCGC TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-6	rev	5' AA GTCGAC GCGGCCGC GGT GTG CAG GTT GAT TTC TGG 3'
	U1-6dupl	for	5' AA TCTAGA TTC AAA TCT TGG TCT CGA GCA AT 3'
	U1-6dupl	rev	5' AA TCTAGA GGT GTG CAG GTT GAT TTC TGG 3'
	U1-7	for	5' AA GTCGAC GCGGCCGC CCG CCA TCT AAT CTT CAA TAG 3'
	U1-7	rev	5' AA GTCGAC GCGGCCGC TCC AGG ATT TTT ACA GTT TTA GT 3'
	U1-8	for	5' AA GTCGAC GCGGCCGC TGG AAT ATA ACC TAA GAA ATA CC 3'
	U1-8	rev	5' AA GTCGAC GCGGCCGC TGT AAT GAA CAT ATT GGT GCA GG 3'
	U1-9	for	5' AA GTCGAC GCGGCCGC GTG CTA TTC TCA CCA GCA AG 3'
	U1-9	rev	5' AA GTCGAC GCGGCCGC TTC CAT TAT ACA GTG TTT AAT TG 3'
lentiviral luciferase reporter system	pRL-con, pRL-U1-UTR	for	5' AA TTCCCGGG CTC ACT ATA GGC TAG CCA CC 3'
	pRL-con	rev	5' AA TTGTCGAC CTA GAA TTA CTG CTC GTT CTT C 3'
	pRL-U1-UTR	rev	5' AA TTGTCGAC CAT TAA CCC TCA CTA AAG GGA A 3'

Table S1 Himmelreich et al.

microRNA	Oligo	Sequence
miR-140-5p	Oligo1	5' GATCCCC**AGTGGTTTTACCCTATGGTAG**TTCAAGAGACTACCATAGGGTAAAACCACTTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAAGTGGTTTTACCCTATGGTAGTCTCTTGAACTACCATAGGGTAAAACCACTGGG 3'
miR-380-3p	Oligo1	5' GATCCCC**TATGTAATATGGTCCACATCT**TTTCAAGAGAAAGATGTGGACCATATTACATATTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAATATGTAATATGGTCCACATCTTTCTCTTGAAAAGATGTGGACCATATTACATAGGG 3'
miR-381	Oligo1	5' GATCCCC**TATACAAGGGCAAGCTCTCTGT**TTCAAGAGAACAGAGAGCTTGCCCTTGTATATTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAATATACAAGGGCAAGCTCTCTGTTCTCTTGAAACAGAGAGCTTGCCCTTGTATAGGG 3'
miR-409-3p	Oligo1	5' GATCCCC**CGAATGTTGCTCGGTGAACCCCT**TTCAAGAGAAGGGGTTCACCGAGCAACATTCGTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAACGAATGTTGCTCGGTGAACCCCTTCTCTTGAAAGGGGTTCACCGAGCAACATTCGGGG 3'
miR-433-3p	Oligo1	5' GATCCCC**ATCATGATGGGCTCCTCGGTGT**TTCAAGAGAACACCGAGGAGCCCATCATGATTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAAATCATGATGGGCTCCTCGGTGTTCTCTTGAAACACCGAGGAGCCCATCATGATGGG 3'
miR-578	Oligo1	5' GATCCCC**CTTCTTGTGCTCTAGGATTGT**TTCAAGAGAACAATCCTAGAGCACAAGAAGTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAACTTCTTGTGCTCTAGGATTGTTCTCTTGAAACAATCCTAGAGCACAAGAAGGGG 3'
miR-650	Oligo1	5' GATCCCC**AGGAGGCAGCGCTCTCAGGAC**TTCAAGAGAGTCCTGAGAGCGCTGCCTCCTTTTTTGGAAA 3'
	Oligo 2	5' AGCTTTTCCAAAAAAGGAGGCAGCGCTCTCAGGACTCTCTTGAAGTCCTGAGAGCGCTGCCTCCTGGG 3'

Table S2 Himmelreich et al.

VII. REFERENCES

1. Cerwenka A, Lanier L. Natural killer cells, viruses and cancer. Nat Rev Immunol. 2001;1:41-49.
2. Robertson M, Ritz J. Biology and clinical relevance of human natural killer cells. Blood. 1990;76:2421-2438.
3. Cooper M, Fehniger T, Caligiuri M. The biology of human natural killer-cell subsets. Trends Immunol. 2001;22:633-640.
4. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.
5. Zamai L, Ponti C, Mirandola P, et al. NK cells and cancer. J Immunol. 2007;178:4011-4016.
6. Herberman R, Ortaldo J. Natural killer cells: their roles in defenses against disease. Science. 1981;214:24-30.
7. Farag S, Caligiuri M. Human natural killer cell development and biology. Blood Rev. 2006;20:123-137.
8. Jacobs R, Hintzen G, Kemper A, et al. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol. 2001;31:3121-3127.
9. Cooper M, Fehniger T, Turner S, et al. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001;97:3146-3151.
10. Fehniger T, Shah M, Turner M, et al. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. J Immunol. 1999;162:4511-4520.
11. Campbell J, Qin S, Unutmaz D, et al. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol. 2001;166:6477-6482.
12. Fehniger T, Cooper M, Nuovo G, et al. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2003;101:3052-3057.
13. Baume D, Robertson M, Levine H, Manley T, Schow P, Ritz J. Differential responses to interleukin 2 define functionally distinct subsets of human natural killer cells. Eur J Immunol. 1992;22:1-6.
14. Miller J, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105:3051-3057.
15. Becknell B, Caligiuri M. Natural killer cells in innate immunity and cancer. J Immunother. 2008;31:685-692.
16. Bryceson Y, March M, Ljunggren H, Long E. Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev. 2006;214:73-91.
17. Farag S, Fehniger T, Ruggeri L, Velardi A, Caligiuri M. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. Blood. 2002;100:1935-1947.
18. Trowsdale J, Barten R, Haude A, Stewart CA, Beck S, Wilson MJ. The genomic context of natural killer receptor extended gene families. Immunol Rev. 2001;181:20-38.
19. Middleton D, Gonzelez F. The extensive polymorphism of KIR genes. Immunology. 2010;129:8-19.
20. Borrego F, Masilamani M, Marusina AI, Tang X, Coligan JE. The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance. Immunol Res. 2006;35:263-278.
21. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci U S A. 1998;95:5199-5204.
22. Sobanov Y, Glienke J, Brostjan C, Lehrach H, Francis F, Hofer E. Linkage of the NKG2 and CD94 receptor genes to D12S77 in the human natural killer gene complex. Immunogenetics. 1999;49:99-105.

23. Le Drean E, Vely F, Olcese L, et al. Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. Eur J Immunol. 1998;28:264-276.
24. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. J Exp Med. 1999;190:1505-1516.
25. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med. 1998;187:2065-2072.
26. Sivori S, Vitale M, Morelli L, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. J Exp Med. 1997;186:1129-1136.
27. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. Immunol Today. 2000;21:228-234.
28. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature. 2001;409:1055-1060.
29. Arnon T, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol. 2001;31:2680-2689.
30. Arnon T, Achdout H, Lieberman N, et al. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. Blood. 2004;103:664-672.
31. Bloushtain N, Qimron U, Bar-Ilan A, et al. Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. J Immunol. 2004;173:2392-2401.
32. Arnon T, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. Nat Immunol. 2005;6:515-523.
33. Cagnano E, Hershkovitz O, Zilka A, et al. Expression of ligands to NKp46 in benign and malignant melanocytes. J Invest Dermatol. 2008;128:972-979.
34. Lakshmikanth T, Burke S, Ali T, et al. NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. J Clin Invest. 2009;119:1251-1263.
35. Nowbakht P, Ionescu MC, Rohner A, et al. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood. 2005;105:3615-3622.
36. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol. 2001;19:197-223.
37. Nausch N, Cerwenka A. NKG2D ligands in tumor immunity. Oncogene. 2008;27:5944-5958.
38. Eagle R, Trowsdale J. Promiscuity and the single receptor: NKG2D. Nat Rev Immunol. 2007;7:737-744.
39. Gilfillan S, Ho E, Cella M, Yokoyama W, Colonna M. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. Nat Immunol. 2002;3:1150-1155.
40. Diefenbach A, Tomasello E, Lucas M, et al. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat Immunol. 2002;3:1142-1149.
41. Diefenbach A, Jamieson A, Liu S, Shastri N, Raulet D. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol. 2000;1:119-126.
42. Groh V, Rhinehart R, Randolph-Habecker J, Topp M, Riddell S, Spies T. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol. 2001;2:255-260.
43. Vivier E, Nunès J, Vély F. Natural killer cell signaling pathways. Science. 2004;306:1517-1519.
44. Guerra N, Tan Y, Joncker N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity. 2008;28:571-580.

45. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science. 1999;285:727-729.
46. Cerwenka A, Bakker A, McClanahan T, et al. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity. 2000;12:721-727.
47. Diefenbach A, Jensen E, Jamieson A, Raulet D. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature. 2001;413:165-171.
48. Cerwenka A, Baron J, Lanier L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci U S A. 2001;98:11521-11526.
49. Jamieson A, Diefenbach A, McMahon C, Xiong N, Carlyle J, Raulet D. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity. 2002;17:19-29.
50. Smyth M, Swann J, Cretney E, Zerafa N, Yokoyama W, Hayakawa Y. NKG2D function protects the host from tumor initiation. J Exp Med. 2005;202:583-588.
51. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature. 2002;419:734-738.
52. Salih H, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood. 2003;102:1389-1396.
53. Coudert J, Zimmer J, Tomasello E, et al. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. Blood. 2005;106:1711-1717.
54. Oppenheim D, Roberts S, Clarke S, et al. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. Nat Immunol. 2005;6:928-937.
55. Ogasawara K, Benjamin J, Takaki R, Phillips J, Lanier L. Function of NKG2D in natural killer cell-mediated rejection of mouse bone marrow grafts. Nat Immunol. 2005;6:938-945.
56. Stern-Ginossar N, Mandelboim O. An integrated view of the regulation of NKG2D ligands. Immunology. 2009;128:1-6.
57. Sutherland C, Chalupny N, Cosman D. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. Immunol Rev. 2001;181:185-192.
58. Eagle R, Traherne J, Hair J, Jafferji I, Trowsdale J. ULBP6/RAET1L is an additional human NKG2D ligand. Eur J Immunol. 2009;39:3207-3216.
59. Chalupny N, Sutherland C, Lawrence W, Rein-Weston A, Cosman D. ULBP4 is a novel ligand for human NKG2D. Biochem Biophys Res Commun. 2003;305:129-135.
60. Cosman D, Müllberg J, Sutherland C, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity. 2001;14:123-133.
61. Radosavljevic M, Cuillerier B, Wilson M, et al. A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. Genomics. 2002;79:114-123.
62. Cerwenka A. New twist on the regulation of NKG2D ligand expression. J Exp Med. 2009;206:265-268.
63. López-Larrea C, Suárez-Alvarez B, López-Soto A, López-Vázquez A, Gonzalez S. The NKG2D receptor: sensing stressed cells. Trends Mol Med. 2008;14:179-189.
64. Schrambach S, Ardizzone M, Leymarie V, Sibilia J, Bahram S. In vivo expression pattern of MICA and MICB and its relevance to auto-immunity and cancer. PLoS One. 2007;2:e518.
65. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci U S A. 1996;93:12445-12450.
66. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein K, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci U S A. 1999;96:6879-6884.

67. Borchers M, Harris N, Wesselkamper S, Vitucci M, Cosman D. NKG2D ligands are expressed on stressed human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2006;291:L222-231.
68. Nowbakht P, Ionescu M, Rohner A, et al. Ligands for natural killer cell-activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias. Blood. 2005;105:3615-3622.
69. Pende D, Rivera P, Marcenaro S, et al. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res. 2002;62:6178-6186.
70. Diermayr S, Himmelreich H, Durovic B, et al. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. Blood. 2008;111:1428-1436.
71. Poggi A, Venturino C, Catellani S, et al. Vdelta1 T lymphocytes from B-CLL patients recognize ULBP3 expressed on leukemic B cells and up-regulated by trans-retinoic acid. Cancer Res. 2004;64:9172-9179.
72. Groh V, Bruhl A, El-Gabalawy H, Nelson J, Spies T. Stimulation of T cell autoreactivity by anomalous expression of NKG2D and its MIC ligands in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2003;100:9452-9457.
73. Hüe S, Mention J, Monteiro R, et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity. 2004;21:367-377.
74. Ogasawara K, Hamerman J, Hsin H, et al. Impairment of NK cell function by NKG2D modulation in NOD mice. Immunity. 2003;18:41-51.
75. Nice T, Coscoy L, Raulet D. Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. J Exp Med. 2009;206:287-298.
76. Jinushi M, Takehara T, Tatsumi T, et al. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. Int J Cancer. 2003;104:354-361.
77. Nomura M, Zou Z, Joh T, Takihara Y, Matsuda Y, Shimada K. Genomic structures and characterization of Rae1 family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. J Biochem. 1996;120:987-995.
78. Venkataraman G, Suciu D, Groh V, Boss J, Spies T. Promoter region architecture and transcriptional regulation of the genes for the MHC class I-related chain A and B ligands of NKG2D. J Immunol. 2007;178:961-969.
79. Gasser S, Orsulic S, Brown E, Raulet D. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. Nature. 2005;436:1186-1190.
80. Yamamoto K, Fujiyama Y, Andoh A, Bamba T, Okabe H. Oxidative stress increases MICA and MICB gene expression in the human colon carcinoma cell line (CaCo-2). Biochim Biophys Acta. 2001;1526:10-12.
81. Nausch N, Florin L, Hartenstein B, Angel P, Schorpp-Kistner M, Cerwenka A. Cutting edge: the AP-1 subunit JunB determines NK cell-mediated target cell killing by regulation of the NKG2D-ligand RAE-1epsilon. J Immunol. 2006;176:7-11.
82. Routes J, Ryan S, Morris K, Takaki R, Cerwenka A, Lanier L. Adenovirus serotype 5 E1A sensitizes tumor cells to NKG2D-dependent NK cell lysis and tumor rejection. J Exp Med. 2005;202:1477-1482.
83. Ebihara T, Masuda H, Akazawa T, et al. Induction of NKG2D ligands on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int Immunol. 2007;19:1145-1155.
84. Schrama D, Terheyden P, Otto K, et al. Expression of the NKG2D ligand UL16 binding protein-1 (ULBP-1) on dendritic cells. Eur J Immunol. 2006;36:65-72.
85. Nedvetzki S, Sowinski S, Eagle R, et al. Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. Blood. 2007;109:3776-3785.

86. Hamerman J, Ogasawara K, Lanier L. Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. J Immunol. 2004;172:2001-2005.
87. Rabinovich B, Li J, Shannon J, et al. Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. J Immunol. 2003;170:3572-3576.
88. Cerboni C, Zingoni A, Cippitelli M, Piccoli M, Frati L, Santoni A. Antigen-activated human T lymphocytes express cell-surface NKG2D ligands via an ATM/ATR-dependent mechanism and become susceptible to autologous NK- cell lysis. Blood. 2007;110:606-615.
89. Langenkamp U, Siegler U, Jörger S, et al. Human acute myeloid leukemia CD34+CD38- stem cells are susceptible to allorecognition and lysis by single KIR-expressing natural killer cells. Haematologica. 2009;94:1590-1594.
90. Armeanu S, Krusch M, Baltz K, et al. Direct and natural killer cell-mediated antitumor effects of low-dose bortezomib in hepatocellular carcinoma. Clin Cancer Res. 2008;14:3520-3528.
91. Dunn C, Chalupny N, Sutherland C, et al. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med. 2003;197:1427-1439.
92. Wu J, Chalupny N, Manley T, Riddell S, Cosman D, Spies T. Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. J Immunol. 2003;170:4196-4200.
93. Stern-Ginossar N, Elefant N, Zimmermann A, et al. Host immune system gene targeting by a viral miRNA. Science. 2007;317:376-381.
94. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe. 2009;5:376-385.
95. Stern-Ginossar N, Gur C, Biton M, et al. Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. Nat Immunol. 2008;9:1065-1073.
96. Schwinn N, Vokhminova D, Sucker A, et al. Interferon-gamma down-regulates NKG2D ligand expression and impairs the NKG2D-mediated cytolysis of MHC class I-deficient melanoma by natural killer cells. Int J Cancer. 2009;124:1594-1604.
97. Bui J, Carayannopoulos L, Lanier L, Yokoyama W, Schreiber R. IFN-dependent down-regulation of the NKG2D ligand H60 on tumors. J Immunol. 2006;176:905-913.
98. Yadav D, Ngolab J, Lim R, Krishnamurthy S, Bui J. Cutting edge: down-regulation of MHC class I-related chain A on tumor cells by IFN-gamma-induced microRNA. J Immunol. 2009;182:39-43.
99. Salih H, Rammensee H, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J Immunol. 2002;169:4098-4102.
100. Kaiser B, Yim D, Chow I, et al. Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands. Nature. 2007;447:482-486.
101. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9:503-510.
102. Ljunggren H, Kärre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.
103. Watzl C, Stebbins CC, Long EO. NK cell inhibitory receptors prevent tyrosine phosphorylation of the activation receptor 2B4 (CD244). J Immunol. 2000;165:3545-3548.
104. Caligiuri M. Human natural killer cells. Blood. 2008;112:461-469.
105. Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cells and dendritic cells: "l'union fait la force". Blood. 2005;106:2252-2258.
106. Smyth MJ, Cretney E, Kelly JM, et al. Activation of NK cell cytotoxicity. Mol Immunol. 2005;42:501-510.
107. http://seer.cancer.gov/statfacts/html/amyl.html.
108. Le Beau MM, Albain KS, Larson RA, et al. Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia:

further evidence for characteristic abnormalities of chromosomes no. 5 and 7. J Clin Oncol. 1986;4:325-345.
109. Davico L, Sacerdote C, Ciccone G, et al. Chromosome 8, occupational exposures, smoking, and acute nonlymphocytic leukemias: a population-based study. Cancer Epidemiol Biomarkers Prev. 1998;7:1123-1125.
110. Moorman AV, Roman E, Cartwright RA, Morgan GJ. Smoking and the risk of acute myeloid leukaemia in cytogenetic subgroups. Br J Cancer. 2002;86:60-62.
111. Smith SM, Le Beau MM, Huo D, et al. Clinical-cytogenetic associations in 306 patients with therapy-related myelodysplasia and myeloid leukemia: the University of Chicago series. Blood. 2003;102:43-52.
112. Nakanishi M, Tanaka K, Shintani T, Takahashi T, Kamada N. Chromosomal instability in acute myelocytic leukemia and myelodysplastic syndrome patients among atomic bomb survivors. J Radiat Res (Tokyo). 1999;40:159-167.
113. von Muhlendahl KE. Chernobyl fallout, nuclear plants and leukaemia: review of recent literature. Eur J Pediatr. 1998;157:602-604.
114. Gundestrup M, Storm HH. Radiation-induced acute myeloid leukaemia and other cancers in commercial jet cockpit crew: a population-based cohort study. Lancet. 1999;354:2029-2031.
115. Yoshinaga S, Mabuchi K, Sigurdson AJ, Doody MM, Ron E. Cancer risks among radiologists and radiologic technologists: review of epidemiologic studies. Radiology. 2004;233:313-321.
116. Sanz GF, Sanz MA, Vallespi T, et al. Two regression models and a scoring system for predicting survival and planning treatment in myelodysplastic syndromes: a multivariate analysis of prognostic factors in 370 patients. Blood. 1989;74:395-408.
117. Horwitz M, Goode E, Jarvik G. Anticipation in familial leukemia. Am J Hum Genet. 1996;59:990-998.
118. Horwitz M. The genetics of familial leukemia. Leukemia. 1997;11:1347-1359.
119. Evans DI, Steward JK. Down's syndrome and leukaemia. Lancet. 1972;2:1322.
120. Bennett J, Catovsky D, Daniel M, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med. 1985;103:620-625.
121. Vardiman J, Harris N, Brunning R. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292-2302.
122. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937-951.
123. Krause D, Van Etten R. Right on target: eradicating leukemic stem cells. Trends Mol Med. 2007;13:470-481.
124. Löwenberg B, Downing J, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341:1051-1062.
125. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev. 2004;18:115-136.
126. Frohling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. J Clin Oncol. 2005;23:6285-6295.
127. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. Annu Rev Genomics Hum Genet. 2002;3:179-198.
128. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat Genet. 1999;23:166-175.
129. Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell. 2002;1:63-74.

130. Grisolano JL, O'Neal J, Cain J, Tomasson MH. An activated receptor tyrosine kinase, TEL/PDGFbetaR, cooperates with AML1/ETO to induce acute myeloid leukemia in mice. Proc Natl Acad Sci U S A. 2003;100:9506-9511.
131. Kelly LM, Kutok JL, Williams IR, et al. PML/RARalpha and FLT3-ITD induce an APL-like disease in a mouse model. Proc Natl Acad Sci U S A. 2002;99:8283-8288.
132. Erickson P, Gao J, Chang KS, et al. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood. 1992;80:1825-1831.
133. Liu P, Tarle SA, Hajra A, et al. Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. Science. 1993;261:1041-1044.
134. Golub TR, Barker GF, Bohlander SK, et al. Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci U S A. 1995;92:4917-4921.
135. Minucci S, Pelicci PG. Retinoid receptors in health and disease: co-regulators and the chromatin connection. Semin Cell Dev Biol. 1999;10:215-225.
136. Warrell RP, Jr., de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med. 1993;329:177-189.
137. Reilly JT. FLT3 and its role in the pathogenesis of acute myeloid leukaemia. Leuk Lymphoma. 2003;44:1-7.
138. Gilliland DG, Griffin JD. Role of FLT3 in leukemia. Curr Opin Hematol. 2002;9:274-281.
139. Knapper S, Burnett AK, Littlewood T, et al. A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. Blood. 2006;108:3262-3270.
140. Lowenberg B, Suciu S, Archimbaud E, et al. Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy--the value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. J Clin Oncol. 1998;16:872-881.
141. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453-474.
142. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood. 2005;106:1154-1163.
143. Cassileth PA, Harrington DP, Hines JD, et al. Maintenance chemotherapy prolongs remission duration in adult acute nonlymphocytic leukemia. J Clin Oncol. 1988;6:583-587.
144. Mayer RJ, Davis RB, Schiffer CA, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. N Engl J Med. 1994;331:896-903.
145. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood. 2001;98:2900-2908.
146. Breems DA, Lowenberg B. Autologous stem cell transplantation in the treatment of adults with acute myeloid leukaemia. Br J Haematol. 2005;130:825-833.
147. Blaise D, Vey N, Faucher C, Mohty M. Current status of reduced-intensity-conditioning allogeneic stem cell transplantation for acute myeloid leukemia. Haematologica. 2007;92:533-541.
148. Aoudjhane M, Labopin M, Gorin NC, et al. Comparative outcome of reduced intensity and myeloablative conditioning regimen in HLA identical sibling allogeneic haematopoietic stem cell transplantation for patients older than 50 years of age with acute myeloblastic leukaemia: a retrospective survey from the Acute Leukemia Working Party (ALWP) of the European group for Blood and Marrow Transplantation (EBMT). Leukemia. 2005;19:2304-2312.
149. Sanz MA, Grimwade D, Tallman MS, et al. Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood. 2009;113:1875-1891.

150. Sievers EL, Larson RA, Stadtmauer EA, et al. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. J Clin Oncol. 2001;19:3244-3254.
151. Kell WJ, Burnett AK, Chopra R, et al. A feasibility study of simultaneous administration of gemtuzumab ozogamicin with intensive chemotherapy in induction and consolidation in younger patients with acute myeloid leukemia. Blood. 2003;102:4277-4283.
152. Stone RM. Induction and postremission therapy: new agents. Leukemia. 2003;17:496-498.
153. Smith BD, Levis M, Beran M, et al. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. Blood. 2004;103:3669-3676.
154. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, et al. Azacitidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia. J Clin Oncol. 2010;28:562-569.
155. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.
156. Diermayr S, Himmelreich H, Durovic B, et al. NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities. Blood. 2008;111:1428-1436.
157. Verheyden S, Demanet C. NK cell receptors and their ligands in leukemia. Leukemia. 2008;22:249-257.
158. Costello RT, Sivori S, Marcenaro E, et al. Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia. Blood. 2002;99:3661-3667.
159. Siegler U, Kalberer CP, Nowbakht P, Sendelov S, Meyer-Monard S, Wodnar-Filipowicz A. Activated natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. Leukemia. 2005;19:2215-2222.
160. Demanet C, Mulder A, Deneys V, et al. Down-regulation of HLA-A and HLA-Bw6, but not HLA-Bw4, allospecificities in leukemic cells: an escape mechanism from CTL and NK attack? Blood. 2004;103:3122-3130.
161. Elkins WL, Pickard A, Pierson GR. Deficient expression of class-I HLA in some cases of acute leukemia. Cancer Immunol Immunother. 1984;18:91-100.
162. Brouwer RE, van der Heiden P, Schreuder GM, et al. Loss or downregulation of HLA class I expression at the allelic level in acute leukemia is infrequent but functionally relevant, and can be restored by interferon. Hum Immunol. 2002;63:200-210.
163. Vollmer M, Li L, Schmitt A, et al. Expression of human leucocyte antigens and co-stimulatory molecules on blasts of patients with acute myeloid leukaemia. Br J Haematol. 2003;120:1000-1008.
164. Verheyden S, Bernier M, Demanet C. Identification of natural killer cell receptor phenotypes associated with leukemia. Leukemia. 2004;18:2002-2007.
165. Verheyden S, Demanet C. Susceptibility to myeloid and lymphoid leukemia is mediated by distinct inhibitory KIR-HLA ligand interactions. Leukemia. 2006;20:1437-1438.
166. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. Blood. 2002;100:1935-1947.
167. Koh CY, Blazar BR, George T, et al. Augmentation of antitumor effects by NK cell inhibitory receptor blockade in vitro and in vivo. Blood. 2001;97:3132-3137.
168. Mazumder B, Seshadri V, Fox PL. Translational control by the 3'-UTR: the ends specify the means. Trends Biochem Sci. 2003;28:91-98.
169. Wilusz CJ, Wormington M, Peltz SW. The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol. 2001;2:237-246.
170. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci. 2001;58:266-277.
171. Mendez R, Richter JD. Translational control by CPEB: a means to the end. Nat Rev Mol Cell Biol. 2001;2:521-529.

172. Chen CY, Del Gatto-Konczak F, Wu Z, Karin M. Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. Science. 1998;280:1945-1949.
173. Klausner RD, Rouault TA, Harford JB. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell. 1993;72:19-28.
174. Shyu AB, Greenberg ME, Belasco JG. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. 1989;3:60-72.
175. Wisdom R, Lee W. The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes Dev. 1991;5:232-243.
176. Trang P, Weidhaas JB, Slack FJ. MicroRNAs as potential cancer therapeutics. Oncogene. 2009;27 Suppl 2:S52-57.
177. Ghildiyal M, Zamore P. Small silencing RNAs: an expanding universe. Nat Rev Genet. 2009;10:94-108.
178. Barbato C, Arisi I, Frizzo ME, Brandi R, Da Sacco L, Masotti A. Computational challenges in miRNA target predictions: to be or not to be a true target? J Biomed Biotechnol. 2009;2009:803069.
179. Cullen BR. Transcription and processing of human microRNA precursors. Mol Cell. 2004;16:861-865.
180. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004;14:1902-1910.
181. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-297.
182. Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004;23:4051-4060.
183. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. 2005;6:376-385.
184. Brodersen P, Voinnet O. Revisiting the principles of microRNA target recognition and mode of action. Nat Rev Mol Cell Biol. 2009;10:141-148.
185. Orom UA, Nielsen FC, Lund AH. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell. 2008;30:460-471.
186. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007;318:1931-1934.
187. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. Cell. 2008;132:9-14.
188. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136:215-233.
189. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9:102-114.
190. Esquela-Kerscher A, Slack F. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006;6:259-269.
191. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6:857-866.
192. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A. 2004;101:2999-3004.
193. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834-838.
194. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002;99:15524-15529.
195. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102:13944-13949.

196. Bonci D, Coppola V, Musumeci M, et al. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med. 2008;14:1271-1277.
197. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008;18:505-516.
198. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64:3753-3756.
199. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005;120:635-647.
200. Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev. 2007;21:1025-1030.
201. Sampson VB, Rong NH, Han J, et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. Cancer Res. 2007;67:9762-9770.
202. Johnson CD, Esquela-Kerscher A, Stefani G, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. 2007;67:7713-7722.
203. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26:745-752.
204. He L, He X, Lim LP, et al. A microRNA component of the p53 tumour suppressor network. Nature. 2007;447:1130-1134.
205. Raver-Shapira N, Marciano E, Meiri E, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell. 2007;26:731-743.
206. Merritt WM, Lin YG, Han LY, et al. Dicer, Drosha, and outcomes in patients with ovarian cancer. N Engl J Med. 2008;359:2641-2650.
207. Karube Y, Tanaka H, Osada H, et al. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. Cancer Sci. 2005;96:111-115.
208. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39:673-677.
209. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature. 2005;435:828-833.
210. Hayashita Y, Osada H, Tatematsu Y, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. 2005;65:9628-9632.
211. Matsubara H, Takeuchi T, Nishikawa E, et al. Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. Oncogene. 2007;26:6099-6105.
212. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A. 2005;102:3627-3632.
213. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci U S A. 2006;103:7024-7029.
214. Gironella M, Seux M, Xie MJ, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. Proc Natl Acad Sci U S A. 2007;104:16170-16175.
215. He LZ, Tolentino T, Grayson P, et al. Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J Clin Invest. 2001;108:1321-1330.
216. le Sage C, Nagel R, Egan DA, et al. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. 2007;26:3699-3708.
217. Fornari F, Gramantieri L, Ferracin M, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene. 2008;27:5651-5661.
218. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res. 2005;65:6029-6033.

219. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A. 2005;102:19075-19080.
220. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006;9:189-198.
221. Shell S, Park SM, Radjabi AR, et al. Let-7 expression defines two differentiation stages of cancer. Proc Natl Acad Sci U S A. 2007;104:11400-11405.
222. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257-2261.
223. Michael MZ, SM OC, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003;1:882-891.
224. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65:7065-7070.
225. Rosenfeld N, Aharonov R, Meiri E, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26:462-469.
226. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. Oncogene. 2007;26:2799-2803.
227. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438:685-689.
228. Elmen J, Lindow M, Schutz S, et al. LNA-mediated microRNA silencing in non-human primates. Nature. 2008;452:896-899.
229. Gentner B, Schira G, Giustacchini A, et al. Stable knockdown of microRNA in vivo by lentiviral vectors. Nat Methods. 2009;6:63-66.
230. Esquela-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. Cell Cycle. 2008;7:759-764.
231. Yu F, Yao H, Zhu P, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007;131:1109-1123.
232. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA Delivery Suppresses Tumorigenesis in a Murine Liver Cancer Model. Cell. 2009;137:1005-1017.
233. Chen C, Li L, Lodish H, Bartel D. MicroRNAs modulate hematopoietic lineage differentiation. Science. 2004;303:83-86.
234. Monticelli S, Ansel KM, Xiao C, et al. MicroRNA profiling of the murine hematopoietic system. Genome Biol. 2005;6:R71.
235. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell. 2007;131:146-159.
236. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature. 2008;451:1125-1129.
237. Chen J, Odenike O, Rowley JD. Leukaemogenesis: more than mutant genes. Nat Rev Cancer. 2010;10:23-36.
238. Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proc Natl Acad Sci U S A. 2008;105:15535-15540.
239. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. Blood. 2008;111:5078-5085.
240. Garzon R, Volinia S, Liu C, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood. 2008;111:3183-3189.
241. Marcucci G, Radmacher MD, Maharry K, et al. MicroRNA expression in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358:1919-1928.
242. Barreau C, Paillard L, Osborne HB. AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res. 2005;33:7138-7150.
243. Chen CY, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci. 1995;20:465-470.

244. Bakheet T, Frevel M, Williams BR, Greer W, Khabar KS. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res. 2001;29:246-254.
245. Pillai R, Bhattacharyya S, Artus C, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005;309:1573-1576.
246. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nat Biotechnol. 2005;23:108-116.
247. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875.
248. Kralovics R, Buser AS, Teo SS, et al. Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. Blood. 2003;102:1869-1871.
249. Salih HR, Antropius H, Gieseke F, et al. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood. 2003;102:1389-1396.
250. Schomber T, Kalberer CP, Wodnar-Filipowicz A, Skoda RC. Gene silencing by lentivirus-mediated delivery of siRNA in human CD34+ cells. Blood. 2004;103:4511-4513.
251. Steinle A, Li P, Morris DL, et al. Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. Immunogenetics. 2001;53:279-287.
252. Aagaard L, Amarzguioui M, Sun G, et al. A facile lentiviral vector system for expression of doxycycline-inducible shRNAs: knockdown of the pre-miRNA processing enzyme Drosha. Mol Ther. 2007;15:938-945.
253. Ramkissoon S, Mainwaring L, Ogasawara Y, et al. Hematopoietic-specific microRNA expression in human cells. Leuk Res. 2006;30:643-647.
254. Yu J, Wang F, Yang G, et al. Human microRNA clusters: genomic organization and expression profile in leukemia cell lines. Biochem Biophys Res Commun. 2006;349:59-68.
255. Meister G, Landthaler M, Dorsett Y, Tuschl T. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA. 2004;10:544-550.
256. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425:415-419.
257. González S, López-Soto A, Suarez-Alvarez B, López-Vázquez A, López-Larrea C. NKG2D ligands: key targets of the immune response. Trends Immunol. 2008;29:397-403.
258. López-Soto A, Quiñones-Lombraña A, López-Arbesú R, López-Larrea C, González S. Transcriptional regulation of ULBP1, a human ligand of the NKG2D receptor. J Biol Chem. 2006;281:30419-30430.
259. Yin W, Xiang P, Li Q. Investigations of the effect of DNA size in transient transfection assay using dual luciferase system. Anal Biochem. 2005;346:289-294.
260. Chae MJ, Sung HY, Kim EH, et al. Chemical inhibitors destabilize HuR binding to the AU-rich element of TNF-alpha mRNA. Exp Mol Med. 2009;41:824-831.
261. Pryzbylkowski P, Obajimi O, Keen JC. Trichostatin A and 5 Aza-2' deoxycytidine decrease estrogen receptor mRNA stability in ER positive MCF7 cells through modulation of HuR. Breast Cancer Res Treat. 2008;111:15-25.
262. Lim L, Lau N, Garrett-Engele P, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005;433:769-773.
263. Sandberg R, Neilson JR, Sarma A, Sharp PA, Burge CB. Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. Science. 2008;320:1643-1647.
264. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004;18:3016-3027.
265. Pauley KM, Eystathioy T, Jakymiw A, Hamel JC, Fritzler MJ, Chan EK. Formation of GW bodies is a consequence of microRNA genesis. EMBO Rep. 2006;7:904-910.
266. Schmitter D, Filkowski J, Sewer A, et al. Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. Nucleic Acids Res. 2006;34:4801-4815.

267. Beitzinger M, Peters L, Zhu JY, Kremmer E, Meister G. Identification of human microRNA targets from isolated argonaute protein complexes. RNA Biol. 2007;4:76-84.
268. Alexiou P, Maragkakis M, Papadopoulos GL, Reczko M, Hatzigeorgiou AG. Lost in translation: an assessment and perspective for computational microRNA target identification. Bioinformatics. 2009;25:3049-3055.
269. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol. 2005;3:e85.
270. Krek A, Grun D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet. 2005;37:495-500.
271. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15-20.
272. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature. 2008;455:64-71.
273. Grosshans H, Filipowicz W. Proteomics joins the search for microRNA targets. Cell. 2008;134:560-562.
274. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008;455:58-63.
275. Easow G, Teleman AA, Cohen SM. Isolation of microRNA targets by miRNP immunopurification. RNA. 2007;13:1198-1204.
276. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature. 2009;460:479-486.
277. Zhang S, Chen L, Jung EJ, Calin GA. Targeting MicroRNAs With Small Molecules: From Dream to Reality. Clin Pharmacol Ther. 2010.
278. Bartkova J, Horejsí Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005;434:864-870.
279. Scott GK, Mattie MD, Berger CE, Benz SC, Benz CC. Rapid alteration of microRNA levels by histone deacetylase inhibition. Cancer Res. 2006;66:1277-1281.
280. Bandres E, Agirre X, Bitarte N, et al. Epigenetic regulation of microRNA expression in colorectal cancer. Int J Cancer. 2009;125:2737-2743.
281. Glozak MA, Seto E. Histone deacetylases and cancer. Oncogene. 2007;26:5420-5432.
282. Kato N, Tanaka J, Sugita J, et al. Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells. Leukemia. 2007;21:2103-2108.
283. Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, Odum N. Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3-dependent expression of MHC class I-related chain A and B. Cancer Res. 2005;65:11136-11145.
284. Soriani A, Zingoni A, Cerboni C, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. Blood. 2009;113:3503-3511.
285. Boissel N, Rea D, Tieng V, et al. BCR/ABL oncogene directly controls MHC class I chain-related molecule A expression in chronic myelogenous leukemia. J Immunol. 2006;176:5108-5116.
286. Armeanu S, Bitzer M, Lauer U, et al. Natural killer cell-mediated lysis of hepatoma cells via specific induction of NKG2D ligands by the histone deacetylase inhibitor sodium valproate. Cancer Res. 2005;65:6321-6329.

Curriculum Vitae

Name and Address:	Heike Himmelreich Hornusserstrasse 12 4600 Olten
Date of birth:	30.05.1979 in Heidenheim a.d. Brenz, Germany
Citizenship:	German

Education

2006 – present	**PhD Thesis** in the Laboratory of Experimental Hematology, Department of Biomedicine, University Hospital Basel, Prof. Dr. Aleksandra Wodnar-Filipowicz und PD Dr. Christian Kalberer
2004	**Diploma** in Biology
2001	**Pre-degree** (Vordiplom) in Biology
1998 - 2004	**Studies of Biology** at the University of Ulm, Germany (Microbiology, Human Genetics, Cell Biology, Analytical Chemistry)
1998	**High School Diploma** (Abitur), Werkgymnasium in Heidenheim a.d. Brenz, Germany

Working Experience

2006 – present	**PhD Student** in the Laboratory of Experimental Hematology, Department of Biomedicine, University Hospital Basel, Prof. Dr. Aleksandra Wodnar-Filipowicz and PD Dr. Christian Kalberer
2004 - 2006	**Research Asscociate** in the Laboratory of Hematology/Oncology, Department of Clinical Research, University Hospital Bern, Prof. Dr. Gabriela Baerlocher
2003 – 2004	**Diploma Thesis** in the Department of Human Genetics, University Hospital Ulm, Germany, Prof Dr. Walter Just Titel: „*Untersuchungen an Kandidatengenen der Geschlechtsdeterminierung nach RDA (Representational Difference Analysis) an Ellobius lutescens DNA*“

Publications

Impact of culture conditions on the proliferative lifespan of human T cells in vitro
Röth A, Schneider L, Himmelreich H, Baerlocher GM, Duhrsen U
Cytotherapy; 2007; 9(1):91-8

Short telomeres and high telomerase activity in T-cell prolymphocytic leukemia (T-PLL)
Röth A, Dürig J, Himmelreich H, Bug S, Siebert R, Dührsen U, Lansdorp PM, Baerlocher GM
Leukemia; 2007; 21(12):2456-62

NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK cell lines with single KIRHLA-class I specificities
Diermayr S, Himmelreich H, Durovic B, Mathys-Schneeberger A, Siegler U, Langenkamp U, Hofsteenge J, Gratwohl A, Tichelli A, Paluszewska M, Wiktor-Jedrzejczak W, Kalberer CP, Wodnar-Filipowicz A
Blood; 2008; 111(3):1428-36

Post-transcriptional regulation of ULBP1 ligand for the activating immunoreceptor NKG2D involves 3' untranslated region
Himmelreich H, Mathys A, Wodnar-Filipowicz A, Kalberer CP
manuscript in preparation, 2010

Oral presentations / posters

NKG2D ligand expression in human B cells is upregulated during lineage differentiation and independent of cellular transformation
Kalberer CP, Diermayr S, Himmelreich H, Wodnar-Filipowicz A
10th Meeting of the Society for Natural Immunity, Cambridge 2007; poster

DNA damage induces upregulation of NKG2D ligand expression in hematopoietic cells
Himmelreich H, Wodnar-Filipowicz A, Kalberer CP
10th Meeting of the Society for Natural Immunity, Cambridge 2007; poster

The recognition of human hematopoietic tumors by NK cells: the role of DNA damage in upregulation of activating NKG2D ligands
Himmelreich H, Wodnar-Filipowicz A, Kalberer CP
20th Meeting of the Swiss Immunology PhD Students, Wolfsberg 2008, oral presentation

Allorecognition of AML blasts and stem/progenitor cells by NK cell lines with single KIR-HLA class I specificities
Kalberer CP, Langenkamp U, Himmelreich H, Diermayr S, Siegler U, Wodnar-Filipowicz A
EFIS-EJI Natural Killer Cell Symposium, Bad Herrenalb 2008, poster

Involvement of microRNAs in post-transcriptional regulation of NKG2D ligand expression in human tumor cell lines
Himmelreich H, Mathys A, Wodnar-Filipowicz A, Kalberer CP
21st Meeting of the Swiss Immunology PhD Students, Wolfsberg 2009, oral presentation

Involvement of microRNAs in post-transcriptional regulation of NKG2D ligand expression in human tumor cell lines
Himmelreich H, Mathys A, Wodnar-Filipowicz A, Kalberer CP
Swiss Society of Hematology (SSH), Basel 2009, oral presentation

Role of microRNAs in post-transcriptional regulation of NKG2D ligand expression in human tumor cell lines
Himmelreich H, Mathys A, Wodnar-Filipowicz A, Kalberer CP
EFIS-EJI Natural Killer Cell Symposium, Freiburg 2009, oral presentation

Scholarship

EFIS-EJI Travel Stipend, EFIS-EJI Natural Killer Cell Symposium, Freiburg 2009